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SYNTHESIS AND COPPER-DEPENDENT ANTIMYCOPLASMAL ACTIVITY OF 3-(2-PYRIDYL)ISOQUINOLINE AND 1,10-PHENANTHROLINE DERIVATIVES

Marcel A.H. de Zwart

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Synthesis and Copper-Dependent Antimycoplasmal Activity of 3-(2-Pyridyl)isoquinoline and 1,10-Phenanthroline Derivatives

VRIJE UNIVERSITEIT TE AMSTERDAM

Synthesis and Copper-Dependent Antimycoplasmal Activity of 3-(2-Pyridyl)isoquinoline and 1,10-Phenanthroline Derivatives

Academisch proefschrift

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Promotor : prof. dr. H. Timmerman
Copromotor : dr. H. van der Goot
Referent : prof. dr. J. K. Seydel

aan mijn ouders
voor Anneke

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CONTENTS

Chapter 1

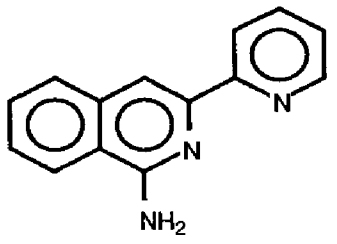
INTRODUCTION TO THE PRESENT INVESTIGATIONS

Previous investigations

Development and (Q)SAR of antimycoplasmal agents containing a 2,2'-bipyridyl moiety

Almost two decades ago Van der Goot described the synthesis of a number of 1-amino-isoquinolines [1]. Microbiological investigations revealed that one of these compounds viz. 1-amino-3-(2-pyridyl)isoquinoline (**1**) was fairly active against *M.gallisepticum.** The presence of a 2,2'-bipyridyl skeleton appeared to be essential with regard to this interesting activity. Furthermore lipophilicity seemed to be a determinant factor for antimycoplasmal activity of these type of compounds.

Based on these findings Pijper started to explore antimycoplasmal activity of compounds in which these requirements are met. So, a number of 1-amino-3-(2-pyridyl)isoquinolines substituted in the benzo ring, and several 3-(2-pyridyl)isoquinolines (**2**) substituted at ortho positions with respect to the nitrogen atoms of the pyridine rings and their 2,2'-bipyridyl, 1,10-phenanthroline and 2-(2-pyridyl)quinoline analogues (**3**, **4** and **5** respectively) were synthesized and tested for their antimycoplasmal activity [2-4]. It was found that in general substitution in the benzo ring had little effect on activity. On the contrary, it was shown for all compounds that substitution at positions adjacent to the nitrogen atoms of the 2,2'-bipyridyl moiety resulted in a remarkable increase of antimycoplasmal activity. Especially introduction of alkyl or alkoxy groups appeared to be very effective in increasing this activity.



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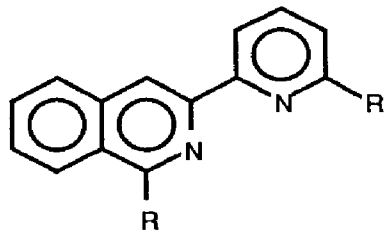

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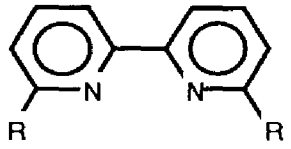

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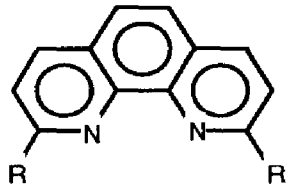

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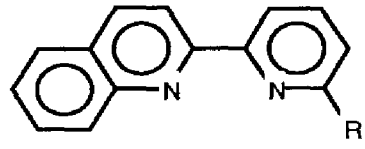

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*See for a short introduction on these microorganisms the addendum to this chapter

During his investigations Pijper encountered a strange phenomenon. All 1-amino-3-(2-pyridyl)isoquinolines were inactive against *M. gallisepticum* in a metal depleted growth medium, but regained their growth inhibiting activity upon addition of a small amount of copper sulfate to that medium. This remarkable effect was studied in more detail by Antic [5,6]. Because 2,2'-biquinoline, 2,9-dimethyl-1,10-phenanthroline (DMP) and compounds alike are known copper(I) chelators it was assumed that also in this case formation of copper complexes might occur. In fact the appearance of $Cu(I)(DMP)_2^+$ and $Cu(I)(Phen)_2^+$ was demonstrated in growing cultures of *M. gallisepticum* [6]. As structural requirements for antimycoplasmal activity were identical with those required for formation of stable copper(I) complexes it was concluded from these studies that the lipophilic copper(I) complexes of these 2,2'-bipyridyl analogues were the active species with respect to antimycoplasmal activity.

Although the activity enhancing effect of ortho substitution has already been mentioned, antimycoplasmal activity of ortho alkyl substituted bipyridyls and phenanthrolines didn't steadily increase with increasing chain length, but reached an optimum in the diethyl derivatives [4]. It is not likely that there is an optimum in complex stability. Because of steric reasons the ortho alkyl substituted bipyridyls and phenanthrolines prefer the tetrahedral configuration of the copper(I) ion (d^{10}) to the square planar or octahedral configuration of the copper(II) ion (d^9). Chain lengthening of the ortho alkyl substituents of these ligands up to three carbon atoms gradually increases the electron density on the donor atoms, resulting in more stable complexes, but further elongation of the alkyl chains has hardly any additional effect on electron density. For this reason the stability of the copper(I) complexes will remain constant.

A for activity optimal value of lipophilicity of these complexes would be more likely. Considering passage of copper complexes across the mycoplasmal cell membrane to be essential in the total sequence of events ultimately leading to growth inhibition, lipophilicity of the complexes has to be sufficiently high but not too high. Very high lipophilicities reduce the tendency of the complexes to 'leave' the membrane.

At that time the qualitative structure-activity relationship originally proposed by Van der Goot could be refined. Besides the presence of copper the following conditions should be fulfilled. In order to possess antimycoplasmal activity compounds structurally related to 2,2'-bipyridyl should be preferably substituted at the carbon atoms adjacent to the nitrogen atoms of the heterocycle. Furthermore cis-coplanarity of the two pyridine rings has to be possible and the compounds should contain a certain degree of lipophilicity.

In addition to this qualitative approach a quantitative structure-activity relationship was established [7]. By multiple regression analysis antimycoplasmal activity, expressed as
- log MIC, was correlated with nine different parameters. Besides the hydrophobic fragmental value several electronic and steric parameters were considered in this study.

Eventually the following equation was obtained by Pijper for a series of 33 congeneric compounds, consisting of eleven ortho-substituted 3-(2-pyridyl)isoquinolines (**2**), eight ortho-substituted 2,2'-bipyridyls (**3**) and fourteen ortho-substituted 1,10-phenanthrolines (**4**), neglecting structures in which appearance of the cis-coplanar conformation of the 2,2'-bipyridyl system is not possible due to steric hindrance:

$$-\log MIC = 2.340\ (\pm 0.355)\ \Sigma_f - 0.309\ (\pm 0.049)\ (\Sigma_f)^2 - 2.739\ (\pm 0.282)\ F^{o,o'} - 0.444\ (\pm 0.093)\ E_s^{o,o'} - 0.387\ (\pm 0.148)\ D_{bipy} - 4.140\ (\pm 0.614)$$

n = 33 r = 0.976 s = 0.200 F = 124

Σ_f : hydrophobic fragmental value calculated according to Rekker[8]

$F^{o,o'}$: inductive/field parameter of Swain and Lupton[9]

$E_s^{o,o'}$: steric parameter of Taft[10]

D_{bipy} : dummy parameter (D_{bipy} = 1 for 2,2'-bipyridyls and D_{bipy} = 0 for 1,10-phenanthrolines and 3-(2-pyridyl)isoquinolines) accounting for possible constant differences between 2,2'-bipyridyls and other compounds considered in this analysis.

The qualitative structure-activity relationship mentioned before is confirmed in many respects by the result of this quantitative approach. It can be deduced from the equation obtained that lipophilicity offers a large contribution to antimycoplasmal activity and furthermore that an optimal lipophilicity exists indeed, although the shape of the maximum is rather flat. The coefficients of both the electronic and steric parameter used, viz. $F^{o,o'}$ and $E_s^{o,o'}$ respectively, are completely compatible with the results of the qualitative structure-activity relationship, in which the activity enhancing effect of ortho alkyl substituents was firmly established.

Although an equation with excellent statistics was obtained, it should be stressed that this quantitative structure-activity relationship is limited to ortho substitited 2,2'-bipyridyls. Furthermore according to this equation antimycoplasmal activity is correlated with parameters belonging to the compounds themselves, whereas the copper(I) complexes are considered to be the active species. Therefore a linear relationship between these parameters and those of the corresponding copper(I) complexes was assumed.

This assumption was verified in a study performed by Gaisser [11]. For a series of five substituted 3-(2-pyridyl)isoquinolines and 2,9-dimethyl-1,10-phenanthroline and their corresponding copper(I) complexes log P values were determined in a 1-octanol/water system. For this rather limited series no linear relationship between log P values of copper(I)

complexes and log P values of the corresponding ligands appeared to exist, indicating that the ligands may have their own role in the mode of action.

Mode of action of 2,2'-bipyridyl related antimycoplasmal agents

Investigations by Smit *et al.* [12,13] showed that various physiological important processes of *Mycoplasma gallisepticum* were inhibited by the copper(I) complex of 2,9-dimethyl-1,10-phenanthroline [$Cu(DMP)_2NO_3$]. The energy yielding metabolism was inhibited because the conversion of pyruvate into lactate was found to be blocked by $Cu(DMP)_2NO_3$, indicating that the enzyme lactate dehydrogenase was inhibited. The minor degradation route of pyruvate to acetate was blocked too, since acetate production ceased shortly after addition of $Cu(DMP)_2NO_3$. The observed decrease of consumption of glucose is a consequence of the inhibition of these metabolic pathways.

Although oxygen consumption was inhibited as well, it seemed not very likely that this is the main effect of this copper complex on *M. gallisepticum* as oxygen consumption is not essential for mycoplasmal growth.

Experiments with crude cell extracts showed an inhibition of NADH oxidase by $Cu(DMP)_2NO_3$ and an even stronger inhibition of NADH oxidase and lactate dehydrogenase by $CuSO_4$. Although it is known that the copper(II) complex of 1,10-phenanthroline and copper(II) ions are able to cause structural changes in the ATP-ase enzyme [14], no strong inhibition of this enzyme in crude cell extracts of *M.gallisepticum* was observed.

Investigations on the influence of $Cu(DMP)_2NO_3$ on DNA, RNA and protein synthesis with growing cells of *M.gallisepticum* showed a selective inhibition of the incorporation of [^{14}C]thymidine into DNA [12]. The fact that $CuSO_4$ is more active in inhibiting enzyme activities in crude cell extracts than $Cu(DMP)_2NO_3$, whereas the opposite is true for the inhibition of cell growth, does suggest that dissociation of the copper complex must take place. This suggestion is confirmed by the significant decrease of the inhibitory effect of both $CuSO_4$ and $Cu(DMP)_2NO_3$ on NADH oxidase upon addition of a small excess of DMP [13].

As $Cu(DMP)_2NO_3$ induced a decrease in the total amount of accessible sulfhydryl groups of whole cells of *M.gallisepticum* , the observed toxicity of $Cu(DMP)_2NO_3$ may be associated with the interaction of copper ions with protein sulfhydryl groups [12].

Uptake studies using ^{67}Cu and [^{14}C]DMP clearly demonstrated that dissociation of $Cu(DMP)_2NO_3$ actually occurred since a much higher Cu:DMP ratio was observed in cell fractions than the ratio of 1:2 in which copper and ligand were added to the growth medium [13]. This was explained by considering copper binding to cell components after dissociation of the complex inside the cell, while the free ligand is distributed between the various compartments (cytosol, membrane, growth medium), leading to an efflux of ligand. The

detection of a significant amount of ^{67}Cu in crude cell extracts which were isolated after incubation of whole cells of *M. gallisepticum* with ^{67}Cu and [^{14}C]DMP and the rapid inhibition of lactate dehydrogenase indicated that copper has been transported across the membrane [13]. Uptake studies by Gaisser [15] revealed that in the presence of copper at minimal inhibitory concentrations of several 2,2'-bipyridyl analogues always the same amount of copper was taken up by the mycoplasma cell. Furthermore it was noticed that in the absence of a ligand, much more copper had to be taken up to cause growth inhibition in comparison with the situation in which a ligand is present. This could be explained by copper binding to the outer surface of the cell membrane, performed by carboxylic or phosphate groups.

Copper uptake appeared to be strongly dependent on the incubation medium used [16]. In phosphate buffered saline without a ligand present a large amount of copper was taken up, whereas in a complex growth medium and in the absence of a ligand hardly any copper entered the mycoplasma cell. This decreased uptake is due to binding of copper to growth medium constituents.

When DMP is present copper uptake is enhanced, both in amount and time. As in the presence of copper also more DMP entered the cell, it was concluded that copper transport was carrier mediated with DMP functioning as carrier [16].

In conclusion the following mode of action of ortho substituted 2,2'-bipyridyl analogues in the presence of copper with regard to antimycoplasmal activity was proposed [11].

Copper, which is bound by growth medium constituents, is chelated by a 2,2'-bipyridyl ligand, which is present at the outer surface of the mycoplasmal cell membrane, due to its hydrophobic character. The resulting copper(II) complex is reduced to the lipophilic copper(I) complex which is subsequently transported across the membrane. After entering the cell, the copper(I) complex dissociates and the free copper is bound by cell components, resulting in inhibition of enzymes involved in the glycolytic pathway viz. NADH oxidase and lactate dehydrogenase.

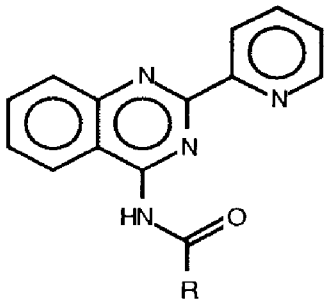
Scope and objectives of the present investigation

Besides vaccines [17] there are many therapeutics of both microbial and synthetic origin for the control of mycoplasmas and treatment of infections caused by them [18-23]. Because of the great number of available antimycoplasmal agents and their many different modes of action it is beyond the scope of this thesis to deal with them all. However, one of the antibiotics which is reported to be effective in treatment of chronic respiratory disease of chickens by *M.gallisepticum* should be mentioned here.

Tylosin [24] is a macrolide antibiotic isolated from a strain of *Streptomyces fradiae* found in soil from Thailand. It is a broad-spectrum antibiotic, interfering with fundamental biological

functions like protein and nucleic acid biosynthesis. In this thesis tylosin is used as a reference compound for antimycoplasmal activity of all new compounds synthesized.

As a broad-spectrum therapeutic like tylosin can induce resistance rapidly, development of new antimycoplasmal agents is currently asked for and accordingly subject of many investigations [25-35]. In this thesis the main topic is the development of more potent antimycoplasmal agents containing the 2,2'-bipyridyl structural element.



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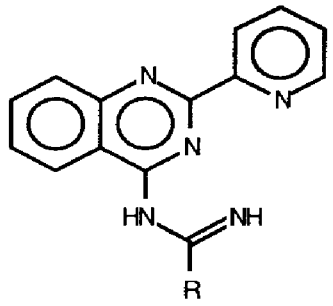

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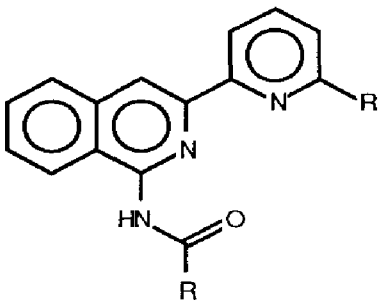
Linschoten *et al.* [36] described the synthesis and antimycoplasmal activity of amides and amidines derived from 4-amino-2-(2-pyridyl)quinazoline (**6** with R = CH_3, C_6H_5, CF_3 and **7** with R = H, CH_3, C_6H_5, CF_3, 2-pyridyl). These compounds appeared to be highly active against *M. gallisepticum* in the presence of copper. To explain this remarkable enhancement of antimycoplasmal activity of these compounds as compared to the starting compound, it was assumed that apart from the nitrogen atoms in the 2,2'-bipyridyl skeleton the unsubstituted amidine nitrogen atom or amide oxygen atom, respectively, might be available for coordination with copper ions. Based on this observation we decided to investigate antimycoplasmal potency of compounds with structural resemblance to the ones described by Linschoten *et al.*



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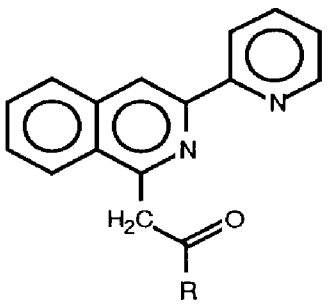

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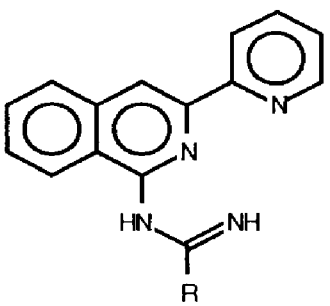

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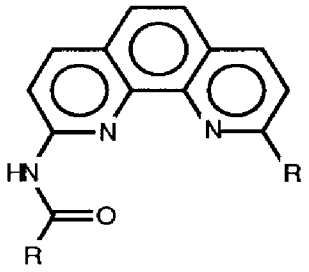

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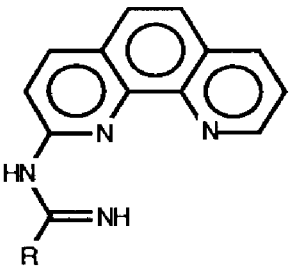

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After a description of several biological activities of copper complexes, synthesis and antimycoplasmal activity of N-[3-(2-pyridyl)isoquinolin-1-yl]amides (**8**), 1-phenacyl-3-(2-pyridyl)isoquinolines (**9**), N-[3-(2-pyridyl)isoquinolin-1-yl]amidines (**10**), N-(1,10-phenanthrolin-1-yl)amides (**11**) and -amidines (**12**) are described in chapters 3, 4, 5 and 6 respectively. In each chapter structure-activity relationships are discussed.

As is clear from previous investigations copper is essential for the growth-inhibitory effect of 2,2'-bipyridyl analogues on *M.gallisepticum*. A subject which has not received much attention sofar, is the possibility that other metals might be able to bring about the same effects. The influence of several other transition metals on both mycoplasmal growth and enzyme activity has therefore been studied and the results are presented in chapter 7.

In accordance with the mode of action in which transport across the lipophilic cell membrane plays an important role, lipophilicity is the major parameter considered for structure-activity relationships. Since formation of a copper complex is also involved in the mode of action, influence of electronic and steric parameters can not be ruled out. Therefore proton association constants of some 2,2'-bipyridyl analogues as well as the stability constants of their copper complexes were determined and are presented in chapter 8. In the same chapter also some speculations on the possible structure of such a copper complex are made.

Addendum

Mycoplasma gallisepticum [22, 23, 37-42]

The test organism used in these investigations is *Mycoplasma gallisepticum* K 514. It is one of the fiftyone presently known species of the genus *Mycoplasma*, classified under the class of Mollicutes. The name of this class, which literally means soft-skin, is derived from the main characteristic of these procaryotes viz. lack of a rigid cell wall. Accordingly, the individual organisms are pleomorphic and vary in shape from coccoid to filamentous to helical. With their diametrical size of 100-1000 nm they are the smallest free-living organisms capable of self-reproduction.

M.gallisepticum is one of the Mollicutes species requiring cholesterol for its growth.

Cholesterol is incorporated into the single trilaminar cell membrane, which is composed mainly of lipids and proteins. The cytoplasm, which is separated from the environment by this membrane, contains ribosomes, RNA and a circular double-stranded DNA molecule. Their genome is small in size (5 x 10^8 Dalton) and low in guanine + cytosine content (23-41%). *M.gallisepticum* is one of the fermentative mycoplasmas in which ATP is generated by glycolysis [43-46]. Glucose is fermented by the Embden-Meyerhof pathway and lactic acid is the major end product formed. Lacking quinones and cytochromes, the respiratory chain is flavin-terminated. Hydrogen peroxide is the end product of respiration in mycoplasmas. When grown aerobically fermentative mycoplasmas oxidize glucose to acetate and carbon dioxide.

Many species of the class Mollicutes are potentially pathogenic, not only for plants and animals but for men as well. It is generally believed that Louis Pasteur first recognized that contagious bovine pleuropneumonia was caused by a specific micro-organism. In subsequent years *Mycoplasma mycoides* var. *mycoides* appeared to be the etiological agent of this rapidly spreading disease. *M.gallisepticum* is the causative agent of chronic respiratory disease in poultry. Upon infection by *M.gallisepticum*, the upper respiratory tract, and particularly the tracheal mucosa, is damaged and gross lesions are found in the air sac. The most common clinical signs include coryza, coughing and sneezing. Besides horizontal infection *M.gallisepticum* can be spread by egg transmission and is, consequently, a hazard to work performed with embryonated chicken eggs or with cell cultures made from such tissue.

M.gallisepticum is unique among mycoplasmas in that it produces toxins which primarily affect the central nervous system, causing encephalitis associated with polyarteritis of cerebral arteries in turkeys. Mortality in infected poultry is relatively rare, and economic loss is caused by reduced production of layers, broiler chickens and turkeys, down grading of carcasses and suboptimal hatchability.

Although pathogenesis of mycoplasmal infections has been the subject of many investigations it is by no means completely elucidated. Nevertheless, hydrogen peroxide, ammonia, exotoxins, neuramidinase and hydrolytic enzymes are regarded as major pathogenic factors. The ability of mycoplasmas to adhere to their host cells assists them in the disease process [47]. The intimate association between adhering mycoplasmas and their host cells provides a situation in which local concentrations of pathogenic factors can build up and cause cell damage.

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Chapter 2

BIOLOGICAL ACTIVITY OF COPPER COMPLEXES AND COPPER CHELATING COMPOUNDS

Introduction

Copper is an essential metallo-element for most, if not all, organisms, being required as an integral part of copper-protein complexes, which often serve as enzymes in performing physiological processes [1]. So, it is not surprising that besides copper itself, copper complexes of organic ligands and copper chelating compounds are able to modulate such activities.

In this chapter a survey is being presented of biologically active compounds of different molecular structure, which have the common property that they owe their biological activity to their ability to bind copper ions. Since the literature available on this topic is rather overwhelming, it is unfortunately impossible to be complete within the framework of this thesis and the attention is mainly focussed on copper complexes and copper chelating agents most frequently encountered in literature.

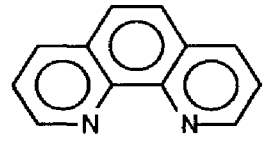
As this thesis is dealing with the influence of copper complexes on growth of *Mycoplasma gallisepticum*, a procaryote lacking a cell wall, special attention is paid to the action of copper complexes against bacteria, fungi and viruses.

Besides this antimicrobial activity, superoxide dismutase activity, anti-inflammatory activity and anticancer activity of different copper complexes will be discussed.

Antibacterial activity

Phenanthrolines

Besides the antimycoplasmal activity of 1,10-phenanthroline derivatives described in chapter 1, various other antibacterial activities of these copper chelating compounds have been reported.



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Investigations of McLeod revealed that 1,10-phenanthroline (**1**) inhibited the growth of lactic acid bacteria [2]. Growth inhibition of these Gram-negative bacteria by this metal-binding agent appeared to be antagonized by several metal ions. However, toxicity was increased in the presence of Cu(II) ions, which is probably due to the formation of a

Cu(II)-1,10-phenanthroline complex. Since the toxicity of 1,10-phenanthroline is not markedly different from that of its non-chelating isomers 1,7-phenanthroline and 4,7-phenanthroline, it was concluded from this study that toxicity of 1,10-phenanthroline was not concerned with its ability to bind metal ions.

The bacteriostatic activities of 1,10-phenanthroline and 2,2'-bipyridine hydrochlorides, quarternary salts and metal chelates on Gram-positive, Gram-negative and acid-fast bacteria have been extensively studied by Dwyer, Shulman and their coworkers [3, 4].

Metal chelates generally are more active than the corresponding ligands and quarternary salts, moreover phenanthroline derivatives are more active than the corresponding bipyridines. For each class of compounds, highest activity was shown against Gram-negative bacteria. This order of sensitivity of bacteria towards metal chelates is more frequently encountered [5, 6].

Except for Ru(II), metal chelates of 5-nitro-1,10-phenanthroline and Ni(II), Fe(II), Co(II), Cu(II), Zn(II), Cd(II) and Mn(II) appeared to be very active against *Mycobacterium tuberculosis*.

Because of its high stability the cation of the Ru(II) chelates as a whole were considered to be the active components. However, activity of the more labile complexes was thought to be a consequence of the base itself or of some metal chelate formed by it with trace metals either in the medium or intracellularly.

Since progressive methylation of each type of compound generally increased antibacterial activity, penetration to or accumulation at vital intracellular sites were considered to be most likely important factors in determining activity. It was found that none of the microorganisms appeared to develop significant resistance to highly active metal chelates.

While useful clinically as topical antimicrobials, selected compounds when administered parenterally were found to be chemotherapeutically ineffective against experimental infection due to *Staphylococcus aureus, Streptococcus pneumoniae* or *Mycobacterium tuberculosis*.

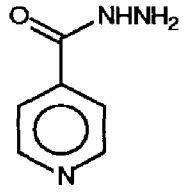
The bacteriostatic activity of phenanthrolines against *Escherichia coli* and *Bacillus subtilis* was studied by Sharrock [6]. 2,9-Dimethyl-1,10-phenanthroline appeared to be the most potent compound against *B. subtilis*, being six times more active than against *E. coli*.

Furthermore, these compounds appeared to be more toxic in the presence of copper(II) with the only exception occuring for *B. subtilis* and 2,9-dimethyl-1,10-phenanthroline. Atomic absorption experiments indicated that phenanthrolines apparently increased the amount of copper which crossed the cell membrane.

Isonicotinic acid hydrazide and other chelating antitubercular drugs [1, 7]

Since 1952, when its action against *Mycobacterium tuberculosis* was first discovered,

isonicotinic acid hydrazide (isoniazid (**2**)) has been one of the most effective agents in tuberculosis therapy.



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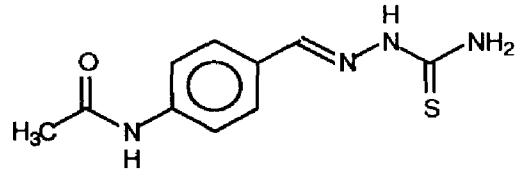

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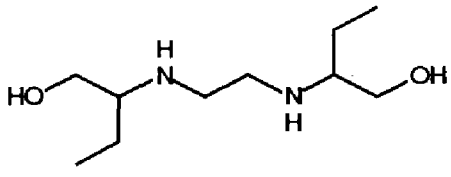
It was clear from the start that the antitubercular action of isoniazid was related to the ability to form chelates, since its activity was enhanced by the presence of copper [8-11]. The enhanced activity of the copper complex was attributed to the increased lipid solubility, which facilitated penetration of the fatty outer membrane of *Mycobacterium tuberculosis*. In a proposed mechanism of action, isoniazid is in fact a pro-drug of the true drug isonicotinic acid, which is N-methylated and built into an analogue of nicotinamide adenine dinucleotide (NAD) [12]. The replacement of NAD as the natural coenzyme by this analogue is held to disturb lipid metabolism.

Also the antitubercular action of thiacetazone (p-acetamidobenzaldehyde thiosemicarbazone (**3**)) is enhanced by copper. Presumably, as with isoniazid, the absorption of the drug depends on chelation. Strains of *Mycobacterium tuberculosis* that have become resistant to isoniazid are still fully sensitive to thiacetazone.



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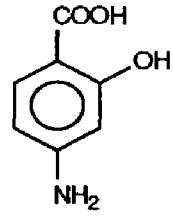

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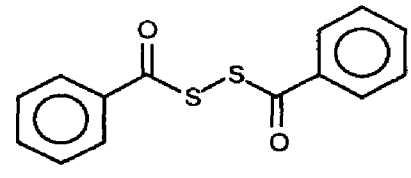

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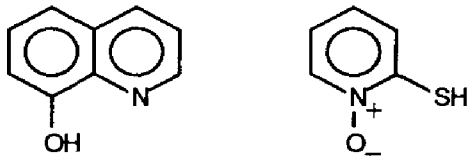
Other metal binding drugs used in treating tuberculosis are the ethylenediamine derivative ethambutol (**4**) and p-aminosalicylic acid (**5**). As the copper(II) complex of the latter was ten times as active as the compound itself, it was suggested that the antitubercular activity of this drug was in fact due to its copper(II) complex. The enhanced activity of the copper complex was attributed to the increased lipid solubility, which facilitated penetration of the fatty outer membrane of *Mycobacterium tuberculosis*.

Also more recent studies have shown the very high antimycobacterial activity of copper chelating agents *in vitro* [13, 14]. Dibenzoyldisulfid (**6**) appeared to be as active as isoniazid against various Mycobacteria *in vitro*.

Antifungal activity

8-Hydroxyquinoline and related compounds [7]

Since 1895, 8-hydroxyquinoline (oxine (**7**)) and its derivatives have been in regular use for topical application in wounds. Besides antibacterial activity 8-hydroxyquinoline appeared to possess antifungal activity as well.



7 **8**

It was shown that 8-hydroxyquinoline exerts its destructive effect on microorganisms through chelation of especially copper and iron. However, antifungal activity was only observed in the presence of cupric ions. In the absence of copper 8-hydroxyquinoline enters the fungal cell, however without causing any harm. Thus it acts not by removing a metal essential for life, but by forming a lethal complex with cupric ions.

Further investigations concerning the mode of action revealed that after the 2:1 8-hydroxyquinoline-copper complex has penetrated the cell membrane, this complex dissociated into the 1:1 complex, which was thought to be the true toxic agent.

Although investigations into the antifungal mechanism of 8-hydroxyquinoline still continue it was assumed that the mode of action of this 1:1 complex in fungi might consist of the oxidative destruction of lipoic acid, which is the essential coenzyme for the oxidative decarboxylation of pyruvic acid [7, 15]. However, disorganization of fungal transport systems has also been proposed as the mode of antifungal action of copper oxinates [15].

It is clear from both proposed mechanisms of actions that a high partition coefficient of 8-hydroxyquinoline and related compounds plays a very important part in determining their action.

Increasing the partition coefficient of 8-hydroxyquinoline didn't appreciably improve the *in vitro* action, whereas a decrease in lipophilicity resulted in a marked reduction of antimicrobial activity. Based on the improved performance against Gram-negative bacteria many halogenated derivatives of 8-hydroxyquinoline have been found therapeutically useful.

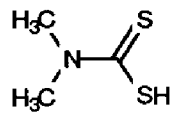
N-oxides of pyridine, quinoline and benzoquinoline are active against micro-organisms provided that an ionizable group is present in the 2-position to make chelation possible. So, 2-mercapto-pyridine-N-oxide (**8**) was found to be antimicrobial to the same extent as 8-hydroxyquinoline.

Although the chelated complex has a somewhat different structure from that of 8-hydroxyquinoline, the mode of action appeared to be the same. It is much used as a fungicide in ointments and soaps and has proved to be very effective against *Pityrosporum ovale*, one of the commonest causes of dandruff.

Compounds containing a NCS or NCO moiety

Dithiocarbamates [7]

Dimethyldithiocarbamic acid (DMDC (**9**)) is a potent fungicide much used in agriculture as its sodium salt. Some agriculturalists prefer to use zinc and iron complexes of DMDC (Ziram and Ferbam respectively), principally because of the excellent adhesion to plants which enables the complexes to resist long periods of rain.



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Others prefer the disulphide (Thiram) obtained by oxidizing DMDC, which is slowly reduced to DMDC under field conditions. Other agricultural fungicides related to DMDC are complexes of ethylene bis(dithiocarbamate) with manganese (Maneb) and zinc (Zineb).

It was shown however, that fungicidal activity of DMDC was actually caused by its copper complex. Due to the extraordinary insolubility of the 1:2 (copper : DMDC) complex in water it was assumed that the 1:1 complex was the ultimate toxic species.

As for 8-hydroxyquinoline, also in this case the mode of action was assumed to be the oxidative destruction of lipoic acid. Toxicity at high concentrations of DMDC has been attributed to an intrinsic toxic effect of the ligand, not related to metal-binding [16].

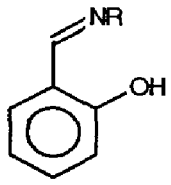
Several thiocarbamate fungicides appeared to have marked antifertility activity in laying hens and mammals as well [17, 18].

Recently it was shown that some of these fungicides inhibited dopamine β-hydroxylase both *in vitro* and *in vivo* by complexing the fully oxidized copper of this enzyme at its active site [19]. The inhibition of dopamine β-hydroxylase appears to be similar in mechanism as the inhibition by disulfiram. Disulfiram has been shown to be non-enzymatically reduced to diethyldithiocarbamate, a potent chelator, which complexes the copper at the enzyme's active site.

Schiff bases, (thio)semicarbazones and related compounds

Various Schiff bases derived from salicylaldehyde (**10**) have been synthesized and tested for

their antifungal activity [20-22]. For all these compounds it was found that fungicidal activity was increased in the presence of transition metal ions. Complex formation appeared to occur through interaction with the azomethine nitrogen and the deprotonated hydroxyl group.



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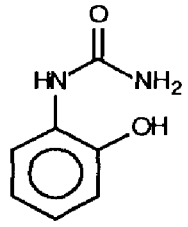

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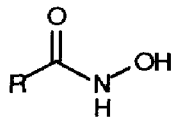

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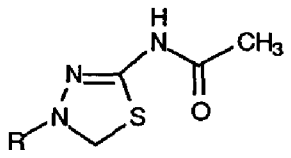
N-(2-hydroxy)phenylurea (**11**) and the aforementioned Schiff bases resemble each other not only in their basic structure but also in their ability of complex formation. As might be expected, it was shown that antifungal activity of N-(2-substituted)phenylureas was also enhanced several times on being coordinated with suitable metal ions like Cu(II), Ni(II), Zn(II), Co(II) and Mn(II) [23].

Another group of compounds containg the NCO moiety and consequently capable of complex formation with transition metal ions is formed by the hydroxamic acids (**12**). Investigation of antifungal activity of diphenylacetohydroxamic acids revealed that all metal chelates were more fungotoxic than their parent ligands [24]. Antifungal activity of these complexes was found to be in the following order: Cu(II) > Ni(II) > Co(II). As the stability of the complex increased, the fungotoxicity was also increased.

In case of 4-substituted 2-acetamido-Δ^2-1,3,4-thiadiazolines (**13**), the NCS moiety is accompanied by an amide function within the same molecule. These compounds and their metal chelates have been tested for their antifungal activity [25]. Also in this case the copper(II) complexes appeared to be more fungotoxic than the free ligands.



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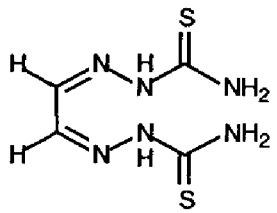

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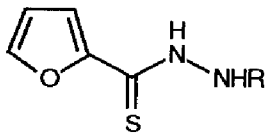

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Besides several other biological activities the antifungal activity of thiosemicarbazone derivatives and their copper(II) chelates have been described [26].

Copper(II) complexes of glyoxal bisthiosemicarbazone (**14**) were more inhibitory to the fungus *Aspergillus flavus* than the ligand. It was suggested that the chelating compound had facilitated the entrance of copper(II) ions into the cell, where these ions might have exerted their toxic effect. Furthermore, N'-substituted derivatives of furan-2-thiocarboxyhydrazide (**15**)

appeared to form deprotonated complexes with several metal ions having 1:1 metal-ligand stoichiometry [27]. Especially copper(II) complexes were found to be highly fungotoxic towards *Aspergillus niger* and *Rhizopus oryzae*, whereas this metal chelate is more fungotoxic than the parent ligand.

Phenanthrolines

Fungostatic properties of phenanthrolines have been described for the first time in 1951 by Blank [28]. 2,9-Dimethyl-1,10-phenanthroline proved to be the most potent fungostatic agent of all phenanthrolines tested against a wide spectrum of pathogenic fungi.

Antifungal activity of some substituted 1,10-phenanthrolines, their quarternary salts and their nickel(II) complexes was investigated by Shulman and Dwyer [3].

Among the nickel complexes investigated, tris(3,5,6,8-tetramethyl-1,10-phenanthroline)-nickel sulfate appeared to be the most active substance against several *Trichophyton* species and *Candida albicans*. Comparison of the activities of 3,5,6,8-tetramethyl-1,10-phenanthroline, its quarternary salt and its tris metal chelate showed that the nickel complex is approximately three times as active as the quarternary salt, whereas the ligand is five times as active as the metal chelate. It was assumed by these investigators that the higher activity of the ligand was due to its greater ability to penetrate to the site of action.

Its capacity to form chelates with essential trace metals at intracellular sites was also considered to be an important factor regulating its activity. In a subsequent study by Shulman *et al.* fungicidal activity of several 1,10-phenanthroline transition metal chelates against a series of pathogenic dermatophytes and *Candida albicans* was investigated [29]. It was shown that hydrochlorides, methiodides as well as fully co-ordinated transition metal chelates of these 1,10-phenanthroline bases were fungicidal for all fungi investigated. In general the methiodides were the least active compounds while the relatively labile Mn(II), Cd(II) and Cu(II) complexes were the most active substances.

In contrast with a previous study by the same investigators, now it was reported that also nickel(II) chelates of 1,10-phenanthroline bases were more active against these fungi than the parent ligand. The relatively inactive divalent metal ions appeared to be toxic in the presence of a chelating agent capable of transporting them into the cell. Furthermore, antifungal activity of transition metal chelates of 1,10-phenanthrolines was not influenced by the nature of the associated anions.

Dependent on the stability of the metal chelate formed the chelate cation as a whole or a derivative of the fully co-ordinated complex, including the dissociated metal or ligand, was assumed to be the active entity. The increase of antifungal activity as a consequence of an increase in lipophilicity of the ligand in the case of *Candida albicans*, suggested that the ability

of the chelate to accumulate at interfaces or penetrate cells is an important factor in the antimicrobial activity of these compounds.

Neither *Candida albicans* nor *Trichophyton mentagrophytes* developed significant resistance to the metal chelates and the hydrochlorides of 3,4,7,8-tetramethyl-1,10-phenanthroline. In a clinical trial, the Mn(II) chelate of 3,4,7,8-tetramethyl-1,10-phenanthroline appeared to be effective in the treatment of chronic dermatological infections due to a variety of dermatophytes or *Candida* species.

The corresponding copper(II) chelate proved to be active in monilial vaginitis where it eradicated the infection in a number of patients who had not previously responded to conventional therapies.

The effects of the zinc concentration of the growth medium on growth and dimorphism of *Candida albicans in vitro* have been analyzed in detail by Bedell et al. [30, 31]. Although the addition of micromolar concentrations of zinc sulfate to the growth medium had no effect on generation time, mycelium formation was strongly suppressed. In the same study the effect of several other divalent cations on fungal growth was investigated. It was found that Co(II) did depress growth rate at micromolar concentrations, whereas Zn(II), Cd(II), Cu(II), Fe(II) and Mn(II) didn't affect growth rate.

Individuals suffering from a hereditary disorder causing reduced zinc levels usually are infected with *Candida*. Zinc suppletion of these patients caused a remission of the symptoms of the defect, including a reduction of *Candida* infection. As fungal growth and formation of invasive mycelium were not suppressed under all conditions by micromolar concentrations of zinc it was considered not very likely that the reduction of *Candida* infection was due to a direct effect of increased zinc at the site of infection. Furthermore, it was shown by Anderson and Soll that zinc-limited cells, when released into fresh medium, take longer to reinitiate protein synthesis because they must reaccumulate zinc to levels necessary to reinitiate RNA synthesis [32].

The influence of several chelators like 1,10-phenanthroline, 8-hydroxyquinoline and EDTA on *Candida albicans* was examined by Bedell and Anderson [33]. The only chelator that was found to inhibit mycelium formation completely and to restrict bud formation to about 10% was 1,10-phenanthroline at minimal concentrations of 50 μM and 230 μM respectively. The inhibition of both phenotypes could be reversed completely by the addition of 200 μM $ZnSO_4$.

It was suggested that the lag period between the release from stationary phase and the onset of development for *Candida* represents the time of acquisition of a minimum threshold amount of a cation, such as zinc.

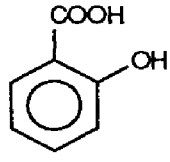
Carboxylates

In order to provide a cheaper alternative for 8-hydroxyquinoline, antifungal efficiency of copper(II) carboxylates was studied [34].

Whereas sodium salts of alkylcarboxylates, mono halo-acetates and arylcarboxylates showed no antimicrobial action against several micobial species, a reasonable degree of antifungal activity was observed for the corresponding copper(II) complexes. As the alkylcarboxylatocopper(II) compounds showed comparatively weak antifungal activity, an impressive increase was observed in the case of copper(II) mono halo-acetates. Activity increased linearly with the size of the halogen substituent. In contrast with this observation, activity of halogen substituted arylcarboxylates was decreased as the size of the halogen substituent increased.

Mixed-ligand complexes

Some investigators have investigated the antimicrobial effect of mixed-ligand or ternary complexes, in which a metal ion is surrounded by two ligand molecules of a different kind [35-39]. By choosing an appropiate pair of ligands either stability or lipid solubility of the resulting complex was modified in order to obtain an increase of antimicrobial activity.



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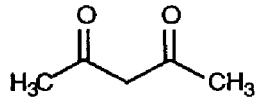

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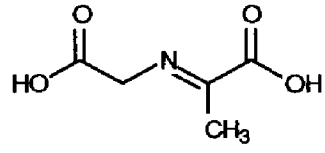

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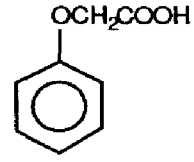

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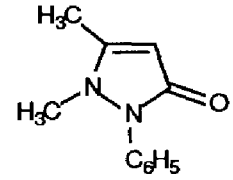

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Mixed complexes of copper(II) with salicylic acid (**16**) and 8-hydroxyquinoline (**7**) or acetylacetone (**17**) appeared to be fairly active against several fungi, whereas the bis (salicylato)copper(II) complex was found to possess very low activity due to its higher hydrophylic character [35, 37]. Ternary complexes of copper(II) with 8-hydroxyquinoline and salicylic acids appeared to have antifungal activity comparable to that of bis(8-hydroxyquinolinato)copper(II), even though the former are less lipophilic [35]. It was assumed that at the site of action dissociation of the mixed complexes had taken place and that the 1:1 cationic complexes thus formed, acted as toxic entities.

Ternary complexes of copper(II) with acetylacetone and various salicylic acids appeared to be better toxic agents than the binary complexes against several fungi [37]. Also in this case it was assumed that penetration of the neutral ternary complex into the cell was followed by dissociaton of this complex at the site of action.

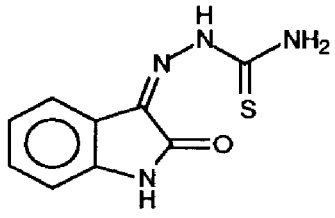
These investigations further confirmed that chelation alone is not sufficient for enhancing antimicrobial activity of a compound but that it must be accompanied by lipid solubility too.

Plesch and coworkers also demonstrated that mixed ligand complexes of copper(II) with N-pyruvidene-glycine (**18**) and several N-donor ligands and copper(II) complexes of aryloxyacetate (**19**) ligands and antipyrine (**20**) were more active against certain fungi than the corresponding binary complexes [38, 39].

Antiviral activity

Thiosemicarbazones [1, 15, 40]

Benzaldehyde thiosemicarbazones were the first synthetic antiviral agents and are active against pox viruses. Structural modifications led to the discovery of the high activity of isatin β-thiosemicarbazone (**21**) and its derivatives, of which methisazone(1-methylisatin β-thiosemicarbazone) is one of the most potent antiviral drugs which has been used clinically in the prevention of smallpox and in the treatment of some of the complications of smallpox vaccination. The fundamental basis for this usage is the observation that the drug inhibits vaccinia virus replication by preventing late viral-protein synthesis. As essential polypeptides are not available and the assembly of viral cores is inhibited, the viral DNA remains uncoated and becomes sensitive to the action of nucleases.



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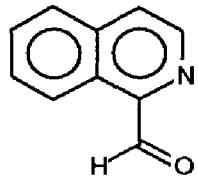

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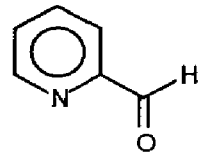

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Furthermore, it was shown that Rous sarcoma virus and other RNA slow viruses, Herpes viruses and arena viruses are inactivated *in vitro* by methisazone. It has been suggested that isatin β-thiosemicarbazones act by metal ion binding, but this has been disputed. Levinson and coworkers [1, 15, 40-42] found that *in vitro* inactivation of several viruses was caused by direct association of methisazone-copper complexes with viral nucleic acids.

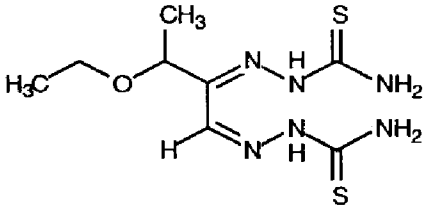
Complexation properties of methisazone and its derivatives have been studied by Stünzi [43, 44]. He showed that isatin β-thiosemicarbazones reduce cupric ion and form copper(I) complexes. Conditional stability constants of these complexes at physiological pH indicate that some *in vivo* complexation of Cu(I) by methisazone may take place. However, in the presence of histidine, a histidinato-copper(II) complex was formed. Furthermore, isatin β-thiosemicarbazones are not expected to form stable complexes with zinc and iron *in vivo*, because stability constants of these complexes are considerably smaller than those of complexes of biologically relevant chelating agents.

Based on these results it was concluded that, although *in vitro* activity of isatin β-thiosemicarbazones may be related to complexation of copper, *in vivo* antiviral action of these compounds is not likely to be the result of metal ion complexation.

Although the involvement of complexes in the antiviral activity of isatin β-thiosemicarbazones is still a matter of debate, the importance of metal ion chelation in the biological activity of two classes of thiosemicarbazones has been firmly established.

Derivatives of pyridine-2-carbaldehyde thiosemicarbazone are tridentate ligands and form stable complexes with copper(II), iron(II), iron(III) and zinc(II) ions. Thiosemicarbazones derived from 1-formylisoquinoline (**22**) and 2-formylpyridine (**23**) and related heterocyclic bases inhibit the growth of DNA viruses of the herpes family and Rous sarcoma virus. The antiviral activity of 2-acetylpyridinethiosemicarbazone observed in an assay of influenza virus RNA dependent RNA polymerase activity might be due to chelation of the zinc ion in the enzyme.

Secondly, the quadridentate bisthiosemicarbazones of α-diketones form very stable copper(II) complexes which are the biologically active species.



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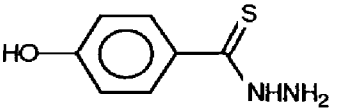

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Kethoxal bisthiosemicarbazone (3-ethoxy-2-oxo-butyraldehyde bisthiosemicarbazone (**24**)) is active against vesicular stomatitis virus in chick embryo cells by inhibiting viral mRNA and protein synthesis, but there is also marked inhibition of cellular DNA, RNA and protein synthesis.

Finally, the copper complex of especially deprotonated 4-hydroxyphenylthiocarboxy-hydrazide (**25**) appeared to be moderately active against Herpes simplex virus [45].

β-Diketones [40]

Many derivatives of acetylacetone are active against Herpes simplex viruses and also some RNA viruses *in vitro*. Complexation of copper and iron seems plausible, but currently the extent of complexation and the mode of action of the β-diketonates are unknown.

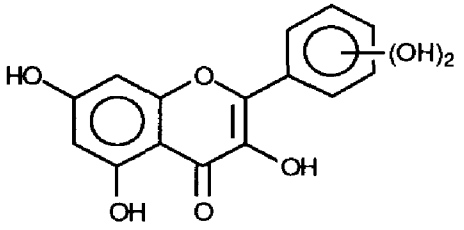
8-Hydroxyquinoline

As the conditional stability constants at pH 7.4 of the copper and zinc complexes of 8-hydroxyquinoline are higher than those of the histidinato complexes, complex formation *in*

vivo can be considered to be certain. It was shown that 8-hydroxyquinoline inhibited RNA-dependent DNA polymerase of Rous sarcoma virus and abolished the ability of this virus to transform cells.

Flavonoids

Flavonoids (**26**) are virucidal to enveloped viruses and have a prophylactic effect against rabies virus in mice. They are also effective against Mengo virus-induced-encephalitis in mice and against vesicular stomatitis virus when tested in cell culture. Complexation of zinc under biological conditions is considered to be likely.



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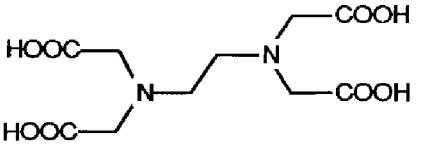

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EDTA

Antiviral activity of EDTA (**27**) is possibly a consequence of the removal of calcium ions bound to the virion. Dissociation of calcium from virions triggers the uncoating process making the viral nucleic acids accessible to attack by nucleases.

Isoniazid [46]

Both cuprous and cupric complexes of isoniazid (**2**) have been shown to possess antiviral activity against Rous sarcoma virus. Moreover, they appeared to be strong inhibitors of both endogenous as well as exogenous reverse transcriptase activity by Rous sarcoma virus. The observed inhibition by these copper complexes was not due to the degradation of the complexes nor was it caused by removal of the integral part of the polymerase by chelation. It was suggested that copper complexes of isoniazid specifically interact with the viral nucleic acids.

Phenanthrolines [47]

A series of doubly charged fully coordinated 1,10-phenanthroline transition metal chelates have been investigated for their ability to inhibit the multiplication of influenza virus in chick allantoic cells *in vitro*. A structure-activity relationship study revealed that the rate of penetration of the active chelate species into the allantoic cell appeared to be an important factor determining virostatic activity. As any sufficiently strong chelating agent seems to inhibit viral and also cellular DNA or RNA polymerases there is no evidence to suggest that these compounds are

potentially valuable antiviral chemotherapeutic agents.

Superoxide dismutase activity

During electron transport in redox reactions, successive reduction of molecular oxygen by single electrons generates a superoxide anion radical, hydrogen peroxide and hydroxyl radical as intermediates [48].

These oxygen-derived free radicals (ODFR) may play a role in the pathogenesis of chronic inflammatory disorders and cancer. In particular, at sites of inflammation, ODFR may amplify inflammatory responses by causing tissue damage through interaction with lipids and a variety of essential macromolecules like proteins and DNA [49].

Although these reactive intermediates can damage living organisms, they are scavenged *in vivo*, in reactions catalyzed by enzymes [48]. Superoxide anions are converted into hydrogen peroxide and molecular oxygen by superoxide dismutase, while hydrogen peroxide in its turn is converted into water and molecular oxygen by catalase. These reactions prevent the formation of the hydroxyl radical, which generally is considered to be the most destructive ODFR.

During the proton dependent and Cu_2Zn_2-superoxide dismutase catalyzed dismutation of superoxide into molecular oxygen and hydrogen peroxide, copper undergoes a redox change from Cu(II) into Cu(I) and subsequently from Cu(I) into Cu(II) [50]. In the active site of the cupric enzyme the Cu(II) is centered in a distorted square planar arrangement coordinated to the nitrogen atoms of four histidine molecules [48, 50].

Copper is the most important metal throughout the catalytic cycle and it remains firmly bound in the active centre [50]. Although this enzyme contains zinc ions too, they don't participate in the catalysis, but they stabilize the active site [48].

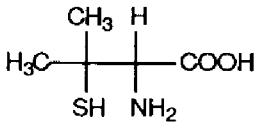
The geometry varies from tetrahedral in the case of Cu(I) to the above mentioned distorted square planar arrangement of the Cu(II) [50]. The protein moiety is flexible enough to allow these geometric changes without losing the metal. So, copper complexes as superoxide dismutase mimicking compounds should be stable in both the I and II form, even under physiological circumstances.

Furthermore, they should be efficient catalysts for superoxide dismutation. Although free copper(II) ions are highly effective as catalysts in the dismutation of superoxide anions, their clinical application is limited because of their toxicity [48].

Furthermore, low molecular weight copper(II) complexes of some amino acids and salicylates appeared to have superoxide dismutating activity [51]. However, in the presence of different chelating agents such as bovine serum albumin or EDTA, this activity is considerably diminished [48, 51, 52].

In this respect it is worth noticing that the copper complex of D-penicillamine (**28**) turned

out to be a very effective dismutating agent [49, 51-53]. As D-penicillamine is used for treatment of Wilson's disease to remove copper excessively deposited in many tissues like brain, kidney and liver, it is clear that D-penicillamine is capable of interactions with copper *in vivo* [51, 52].



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The high stability of the copper complex of D-penicillamine permits the supposition that such a copper complex should also show superoxide dismutating activity *in vivo* [52].

D-penicillamine is widely employed as an antirheumatic agent and it is entirely possible that it owes its beneficial effect in patients with rheumatoid arthritis to its ability to form a copper complex *in vivo* with superoxide dismutating properties [52, 53].

Antiinflammatory activity

Among the many pharmacological activities of copper complexes, the antiinflammatory activity is one of the most extensively studied. Some excellent reviews about this topic written by Sorenson have been published recently [1, 54-58].

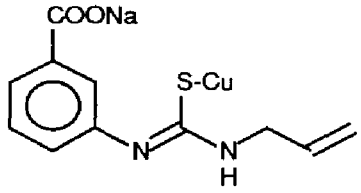
Studies of copper concentrations in tissues from individuals with rheumatoid arthritis have revealed that these concentrations are altered in this disease state. Compared to normal healthy individuals, patients with rheumatoid arthritis have higher mean serum or plasma copper concentrations, which are directly related to disease severity. Furthermore, it was found that the increased rate of synthesis and the accelerated turnover rate of ceruloplasmin, a copper containing metallo-enzyme, was directly related to disease activity. Copper was also found to be increased in synovial fluid of these patients and it was suggested that ceruloplasmin, which accounted for most of the rise in synovial fluid copper, increased with increasing duration of disease.

All these processes involved with the modification of copper metabolism can be interpreted as part of a multifaceted physiological response to inflammatory disease.

With regard to altered concentrations of copper in arthritis, it is of special interest to note that a number of copper-dependent enzymes are required for the protection against tissue damage and for the repair of inflamed tissue. These include cytochrome-c oxidase, ceruloplasmin, lysyl oxidase and superoxide dismutase.

One of the first copper complexes reported to possess antiinflammatory activity was sodium 3-(N-allylcuprothiouredo)-1-benzoate (Cupralene (**29**)), a compound used in the treatment of tuberculosis. From that time occasional publications reported the antiinflammatory activity of

cuprous iodide, cupric carbonate and cupric complexes of several carboxylic acids like salicylic acid (**16**) in various models of inflammation.



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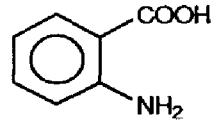

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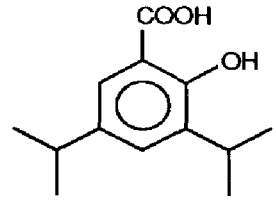

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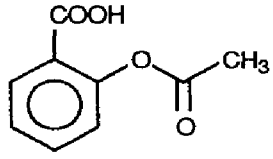
Copper chelates of both anthranilic acid (**30**) and 3,5-diisopropylsalicylic acid (**31**) appeared to be very much more active in three different animal models of inflammation than either cupric acetate or the respective parent ligand [54]. It was concluded from this study that the observed antiinflammatory activity is mediated by the intact chelate.

In addition to the marked increase in antiinflammatory activity, a reduced toxicity of these chelates as compared with the parent ligands was found. Of even greater interest was the observation that these chelates were potent antiulcer compounds. Since it is well known that clinically used antiarthritic drugs cause ulcers and gastrointestinal distress, the observed antiulcer activity further distinguishes these coordination compounds from their parent compounds.

As copper complexes of many non-antiinflammatory complexing agents appeared to have antiinflammatory activity in animal models of inflammation, it was suggested that copper complexes of clinically used antiarthritic drugs were formed *in vivo* and that they were responsible for the beneficial antiarthritic effects of these drugs.



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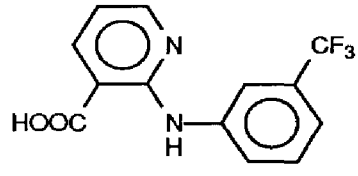

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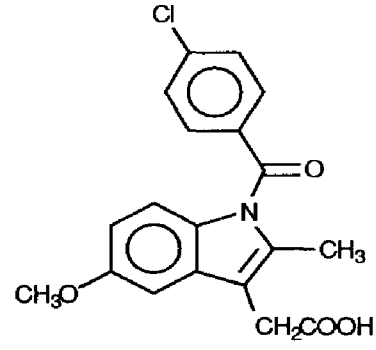

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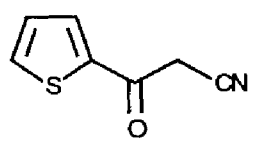
In addition, copper complexes of antiarthritic drugs, including salicylic acid (**16**), acetylsalicylic acid (**32**), niflumic acid (**33**), D-penicillamine (**28**), indomethacin (**34**) and several corticoids were found to be more active than the parent drugs.

Furthermore, a number of other copper complexes have also been found to have

antiinflammatory activity. For instance, the copper(II) complex of β-oxo-2-thienylpropionitrile (**35**) appeared to be effective in treating rat polyarthritis.

Also the copper complex of glycyl-L-histidyl-L-lysine (**36**) is claimed to have anti-inflammatory activity and increase the rate of wound healing.

Whereas the copper complexes of the two stereo-isomers of histidine (**37**) were found to be active in different inflammatory models, their parent ligands were ineffective [59].



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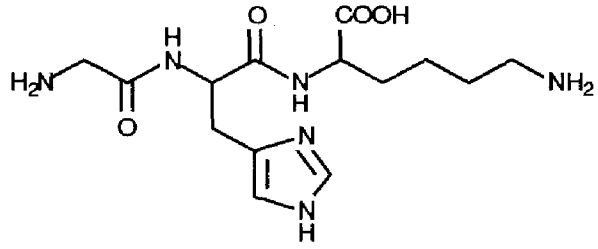

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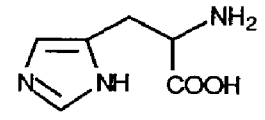

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So, not only copper complexes of non-antiinflammatory complexing agents appeared to have antiinflammatory activity, but also copper complexes of antiarthritic drugs have been found to be more active or more effective antiinflammatory agents than the parent drugs.

As a result of intensive investigation, the knowledge of the possible biochemical needs for copper during inflammation has increased.

At this moment a summary of the plausible biochemical mechanisms includes: induction of lysyl oxidase, modulation of prostaglandin synthesis, induction of superoxide dismutase or superoxide dismutase-mimetic activity, stabilisation of lysosomal membranes and induction or stabilisation of various enzymes involved in tissue repair processes.

Anticancer activity

3,5-Diisopropylsalicylate

In various types of cancers increased copper concentrations in serum or plasma have been found. Furthermore, it was shown that all tumour cell lines have markedly decreased superoxide dismutase activity. As a result of this observation and the known superoxide dismutase-mimetic activity of copper salicylate complexes, salicylate and other copper complexes were investigated as antitumour agents.

It was found that within a series of salicylates the lipid soluble copper(II) complex of 3,5-diisopropylsalicylic acid (**31**) was the most effective antitumour complex [1].

In addition to being effective against sarcoma 180 tumours this copper complex also inhibited growth of solid Ehrlich carcinomas and markedly increased the life span of mice bearing this solid tumour. Increasing the number of treatments further increased survival time

by inhibiting metastasis and decreasing tumour growth.

Bleomycin [60]

The bleomycins are a family of glycopeptide derived antibiotics that have been used clinically against certain malignant lymphomas and squamous cell carcinomas.

The therapeutic activity of bleomycins is generally believed to correlate with their ability to bind to and degrade DNA. It was establised that DNA strand scission requires molecular oxygen and a metal ion, with Fe(II) being the most extensively studied and most active.

The total structure of bleomycin can be divided into three domains, with each its specific function. Domain 1 is involved in formation of the metal complex. Although the nature of the ligands and their arrangement in the iron complexes still remain controversial, copper is square-pyramidal surrounded by three nitrogen atoms of primary and secondary amines of β-aminoalanine moieties and by the nitrogen atoms of a pyrimidine and imidazole nucleus.

Domain 2 is required for DNA binding and sequence specific recognition, while domain 3 may be responsible for the selective accumulation of bleomycin in some cancer cells.

As the presence of Fe(II)ions and O_2 appeared to be essential requirements for the bleomycin mediated degradation of DNA *in vitro*, it was proposed that active oxygen radical species may be responsible for this degradation. Although it was shown that both hydroxyl radicals and superoxide anion radicals are generated by ferrous bleomycin, evidence was provided against the involvement of these species in the DNA degradation process.

Based on mechanistic studies it is now generally believed that DNA cleavage by bleomycin involves the fragmentation of the deoxyribose backbone by radical processes.

Copper(II) bleomycin and metal free bleomycin can inhibit the growth of tumour cells in tissue culture and tumour growth in animals. These results contrast dramatically with *in vitro* studies which have shown the inability of copper bleomycin to degrade DNA, the molecular mechanism thought to be responsible for its cytotoxicity *in vivo*.

To explain for this remarkable difference two models have been proposed. One model considers Cu(I) a direct participant in the oxidative DNA damage, whereas the other model considers Cu-bleomycin as a prodrug, delivering bleomycin to the nucleus where it then becomes a spectator, and Fe(II) is responsible for oxidative damage.

Although sound evidence is provided in favour of both models, at present the role of Cu vs Fe bleomycin as mediators of *in vivo* damage remains unanswered.

Phenanthrolines

Interest in the antineoplastic activities of phenanthroline copper complexes commenced following reports that the copper(II) complex of 3,4,7,8-tetramethyl-1,10-phenanthroline had

antitumour activity in rodents [61]. Furthermore, the kinetic inert Ru(II) and Ni(II) complexes of the same ligand appeared to be less active against P388 mouse lymphocytic leukaemic cells than the corresponding more labile chelates of Cu(II), Cd(II), Zn(II), Fe(II) and Co(II). However, neither the Cu(II) nor the Ru(II) chelates significantly increased the survival time of mice inoculated with these tumour cells [62].

Falchuk and Kirshan investigated the influence of 1,10-phenanthroline on lympoblast cell proliferation [63]. It was found that lymphoblast cell growth was completely inhibited by 1,10-phenanthroline at micromolar concentrations. However, proliferation of lymphoblasts was unaffected by the non-chelating isomer 1,7-phenanthroline, indicating that the 1,10-phenanthroline effects are most probably mediated through its metal-chelating properties. It was found that the inhibition of cell proliferation by 1,10-phenanthroline could be reversed by the addition of Zn(II), Cu(II) or Fe(II).

With respect to the *in vivo* situation, it is worth noticing that 1,10-phenanthroline has been observed to complex with copper and iron in several tissues [18]. Although inhibition of proliferation could proceed through a number of biochemical modes, it was suggested that the mode of 1,10-phenanthroline inhibition was the formation of a chelate-metal-enzyme complex, affecting the transition of different cell cycles.

In addition, a number of investigations have revealed the oxidative nuclease activity of the 1,10-phenanthroline-cuprous complex with hydrogen peroxide as coreactant in a model cell-free system [60,64-69]. In a proposed mechanism of action the copper(II) complex was reduced by superoxide anions, resulting in the formation of the copper(I) complex and oxygen. As superoxide anions can also undergo a dismutation reaction to generate hydrogen peroxide, both cofactors are produced, which then give rise to the formation of hydroxyl radicals, which are responsible for the oxidative damage of DNA.

Whereas copper(II) ions antagonized the inhibitory action of 1,10-phenanthroline on lymphoblast cell proliferation, 2,9-dimethyl-1,10-phenanthroline appeared to be a potent cytotoxin against L1210 cells *in vitro* only in the presence of copper [70].

Thiosemicarbazones

Thiosemicarbazones were among the first antineoplastic compounds studied in which copper(II) was shown to enhance ligand activity. One of the most active compounds was 3-ethoxy- 2-oxobutyraldehyde bisthiosemicarbazone (H_2KTS (**24**)) which proved to be a superior chemotherapeutic agent against several rat tumours [61]. It was also found that copper(II) enhanced the cytotoxicity of H_2KTS against several tumour cells in suspension. By itself, H_2KTS had insignificant cytotoxicity *in vitro* using these systems. Supporting these observations, the pronounced *in vivo* antitumour activity of Cu(II)KTS was established.

After reports that copper enhanced the cytotoxicity of H_2KTS, a number of investigators presented information that established the unique role that copper(II) had on the antitumour action of H_2KTS *in vivo*, and the formation of Cu(II)KTS as the active metabolic intermediate. It was shown that the complex as a whole was the active species. It is not clear however what the mode of action of this chelate is, although a number of plausible mechanisms have been suggested.

Interference of CuKTS with the incorporation of thymine into DNA was one of them.

Another possible mechanism of action suggested the reduction to the cuprous state by thiols [71]. The cuprous complex, once formed intracellularly, rapidly reacts with other thiols in an exchange reaction to inhibit DNA synthesis and oxidative phosphorylation.

Although thiosemicarbazones may exhibit antineoplastic activity by interacting with DNA or by preventing its synthesis, care should be taken when following this line of thought since both ZnKTS and $ZnCl_2$ inhibit DNA synthesis but neither is known to have antineoplastic activity [61].

In addition to bisthiosemicarbazones some monothiosemicarbazones have been examined for antineoplastic activity [61]. It was reported that thiosemicarbazones of the N-heterocyclic carboxaldehydes **22** and **23** had no deleterious effect on cell survival and tumour growth, whereas both iron(II) and copper(II) complexes substantially inhibited cell proliferation in mice, with copper(II) complexes being much more effective.

Among the important mechanistic conclusions emanating from the structural studies was the finding that an N-N-S tridentate ligand system is a common feature of all compounds with carcinostatic potency. As metal complexes of 2-formylpyridine thiosemicarbazones have both *in vitro* and *in vivo* activity against tumour cells, studies concerning the mechanism of action of these compounds were mainly aimed at metal binding.

The principal site of inhibition appeared to be the enzyme ribonucleotide reductase, which catalyzes a critical and possibly rate limiting step in DNA synthesis and cell division [72] and was inhibited *in vitro* by iron complexes of these compounds.

In addition, thiosemicarbazone ligands are known to sequester iron *in vivo* from the host organisms and the copper complex of 2-formylpyridine thiosemicarbazone is also stable in biological systems. The reaction of the copper(II) chelate of 2-formylpyridine thiosemicarbazone with cells appeared to produce several effects: *i* the copper chelate is bound to cellular ligands, perhaps glutathione, *ii* cellular thiol groups are oxidized and*iii* reduced oxygen radicals are generated.

Also incorporation of thymidine into DNA was inhibited to the same extent as was found for CuKTS.

Hence, there are multiple biological responses to these copper complexes and it is still unclear how the initial chemical reactions relate to irreversible cytotoxicity.

Schiff bases of salicylaldehyde

Another important and most promising group of antitumour agents is formed by Schiff bases of salicylaldehyde (**10**). Elo and Lumme reported on the antiproliferative activity of trans-bis(salicylaldoximato)copper(II) and related chelates [73-77]. These compounds were shown to totally arrest the proliferation of tumour cells *in vitro* and to have a powerful activity against Ehrlich ascites carcinoma *in vivo*. Antiproliferative activity was only observed for the intact chelates. From the other transition metals studied only the cobalt analogue had intermediate activity, whereas the other metal chelates were inactive [76].

Because salicylaldoxime resembles pyridoxal oxime, an extremely powerful inhibitor of pyridoxal kinase, it was supposed that pyridoxal antagonism might be involved in the mechanism of action of these chelates. However, there was no direct evidence for this theory and the drastically different antiproliferative activities of the various metal chelates was hard to explain on the basis of this theory alone.

Recent investigations revealed a distinct correlation between antiproliferative activity and reactivity of trans-bis(salicylaldoximato)copper(II) with thiols, especially glutathione [75]. Thus, the mechanism of action might be similar to that proposed for the copper(II) chelates of bisthiosemicarbazones, involving the formation of catalytically active Cu(I) compounds in the reaction with cellular thiols and subsequent oxidation of further cellular thiols.

Salicylaldehyde benzoylhydrazones are also Schiff bases of salicylaldehyde with antitumour properties [61, 78-80]. These compounds appeared to be potent inhibitors of DNA synthesis in a variety of cultured human and rodent cells, and their Cu(II) complexes produce significant inhibition of tumour growth when given to mice bearing a transplanted fibrosarcoma. Their cytotoxicity is equal to or greater than that of many chelators previously known to possess such properties, including compounds used clinically. The Schiff bases of salicylaldehyde mentioned above are relatively non-toxic to mice and show some selectivity in their effects on different cell types.

Conclusions

Among the many biological activities of copper complexes antimicrobial activity, superoxide dismutase activity, anti-inflammatory activity and anticancer activity are most frequently encountered in literature. In general, the biological activity of copper chelating compounds is enhanced in the presence of copper. This is a consequence of the increased lipid solubility of the copper complex as compared to the parent ligand. Transport of both metal and ligand across

lipophilic membranes to vital intracellular sites is favoured by chelation. Once intracellular, the fully co-ordinated complex or one of its derivatives, including the dissociated metal or ligand, may be the active entity.

Copper chelating compounds included in this survey are 1,10-phenanthrolines, 2,2'-bipyridyls, 8-hydroxyquinoline, compounds containing a NCO or NCS moiety like hydroxamic acids, hydrazides, thiosemicarbazones and dithiocarbamic acids, carboxylic acids and Schiff bases of salicylaldehyde.

Many copper chelating agents are known to exert an inhibitory action on protists and viruses. The antibacterial activity of 1,10-phenanthrolines against both Gram-negative and Gram-positive bacteria is enhanced in the presence of copper. Gram-negative bacteria appeared to be more sensitive towards these copper complexes than Gram-positive bacteria.

In case of the antimycobacterial activity of the copper complex of isonicotinic acid hydrazide, the ligand appeared to be the ultimate active species.

Copper complexes of dithiocarbamates and 8-hydroxyquinoline exert their antifungal effect by oxidative destruction of a thiol containing essential coenzyme. Antifungal activity of copper complexes of thiosemicarbazones, Schiff bases of salicylaldehyde and other compounds containing an NCO or NCS moiety is a consequence of the toxic effect of copper(II) ions, which entrance into the cell is facilitated by the chelating compound.

Antiviral activity of 1-methylisatin β-thiosemicarbazone and 3-ethoxy-2-oxo-butyraldehyde bisthiosemicarbazone is probably caused by the inhibition of viral protein synthesis by the respective copper complexes.

Superoxide dismutase is a copper containing enzyme which catalyzes the dismutation of superoxide anions into molecular oxygen and hydrogen peroxide. During this dismutation Cu(II) is initially reduced to Cu(I), which is subsequently oxidized to Cu(II). The copper complex of D-penicillamine appeared to be a very effective superoxide dismutating agent.

Among the many pharmacological activities of copper complexes, the anti-inflammatory activity is one of the most extensively studied. Anti-inflammatory activity of copper complexes is a consequence of the increased plasma copper concentration. This leads to the induction of various copper-dependent enzymes involved in tissue repair processes. Furthermore, it is suggested that many anti-inflammatory drugs owe their beneficial effect to copper complexation.

Copper complexes appear to possess anticancer activity too. The anticancer activity of the copper(II) complex of 3,5-diisopropylsalicylic acid is ascribed to its superoxide dismutase-mimetic activity, because all tumour cell lines have markedly decreased superoxide dismutase activity.

The copper complex of the antibiotic bleomycin owes its anticancer activity to its ability to

bind and to degrade DNA. Although the role of copper in the whole process is not clear, it is generally believed that DNA cleavage by bleomycin involves the fragmentation of the deoxyribose backbone by radical processes.

Lymphoblast cell proliferation was inhibited by 1,10-phenanthroline and it was suggested that the mode of inhibition was the formation of a chelate-metal-enzyme complex, affecting the transition of different cell cycles.

Furthermore, the oxidative nuclease activity of the 1,10-phenanthroline-cuprous complex with hydrogen peroxide as coreactant was established in a model cell-free system.

At last, it was reported that 3-ethoxy-2-oxo-butyraldehyde bisthiosemicarbazone, thiosemicarbazones of α-N-heterocyclic carboxaldehydes and Schiff bases of salicylaldehyde possess antineoplastic activity in the presence of copper. One of the possible mechanisms of action proposed, consists of the formation of catalytically active Cu(I) compounds in the reaction with cellular thiols and the generation of reduced oxygen radicals.

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Chapter 3

SYNTHESIS AND COPPER-DEPENDENT ANTIMYCOPLASMAL ACTIVITY OF 1-AMINO-3-(2-PYRIDYL)ISOQUINOLINE DERIVATIVES.1.AMIDES*

Marcel A.H. de Zwart, Henk van der Goot and Henk Timmerman

Abstract In order to investigate the antimycoplasmal activity of compounds structurally related to 2,2'-bipyridyl a series of both aliphatic and aromatic amides derived from 1-amino-3-(2-pyridyl)isoquinoline were synthesized. The most active compounds appeared to be as active as tylosin, an antimycoplasmal therapeutic which is used in veterinary practice, in the presence of a small non-toxic amount of copper. Furthermore it was found that antimycoplasmal activity depends on the hydrophobic fragmental value of the amide residue. A quantitative structure-activity relationship established the optimal hydrophobic fragmental value of the amide residue to be 0.30.

Introduction

Mycoplasmas are known to be causative agents of many infectious diseases not only in plants and animals but in man as well [1-3]. Broad-spectrum antibiotics from the small polyene type (34 to 37 carbon atoms) and the tetracycline type are inhibitory to mycoplasmas *in vitro* as well as *in vivo*. Unfortunately these broad-spectrum antibiotics induce resistance rapidly [1]. Tylosin, a macrolide antibiotic is often used in therapy of mycoplasmal infections in poultry [1].

Furthermore Pijper *et al.* [4] have shown that in the presence of a small and non-toxic amount of copper certain 2,2'-bipyridyl derivatives are highly active against mycoplasmas. Due to the low activity of these compounds in the absence of copper it was concluded that growth inhibition is caused by their copper complexes rather than by these compounds themselves. In a study on the mechanism of action of these copper complexes Smit *et al.* [5] and Gaisser *et al.* [6,7] discovered that copper itself is the ultimate toxic agent, whereas ligands facilitate copper transport across the membrane through the formation of lipophilic complexes.The toxicity of copper is most probably based on the inhibition of enzymes involved in the energy providing metabolism like NADH-oxidase and lactate dehydrogenase [7].

In a recent study from our laboratory Linschoten *et al.* [8] reported on the antimycoplasmal activity of a series of amides and amidines derived from 4-amino-2-(2-pyridyl)quinazoline. It was found that the most active compound N-[2-(2-pyridyl)quinazolin-4-yl]-2-pyridinecarboxamidine was on a molar basis forty times as active as tylosin, which was used as a reference compound. Based on these results we decided to continue our search for new antimycoplasmal

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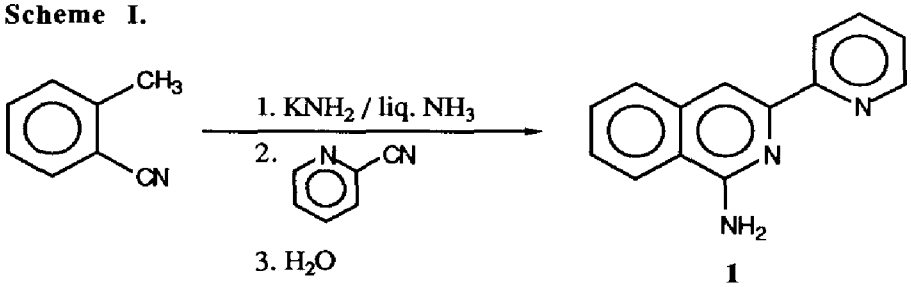
therapeutics with the synthesis of amides and amidines derived from 1-amino-3-(2-pyridyl)-isoquinoline (**1**), which has certain advantages over the structurally related 4-amino-2-(2-pyridyl)quinazoline from a synthetic point of view .

In the present paper we report on the synthesis and antimycoplasmal activity of both aliphatic and aromatic amides derived from 1-amino-3-(2-pyridyl)isoquinoline. In order to establish the structure with optimal activity we used the efficient method proposed by Topliss [9]. This method is an application of the Hansch approach and is based on a proper selection of an initial small group of compounds. Analysis of the potency order provides a rational basis for the selection of more potent analogues.

Chemistry

A general method for the synthesis of amides consists of the acylation of amines by agents like acyl chlorides [10]. While these acyl chlorides can be obtained from the corresponding acids very easily [11,12], our major concern was the synthesis of the required amine. Although the synthesis of 1-amino-3-(2-pyridyl)isoquinoline has been described, the yields are rather poor (25-28%) [13,14]. Therefore we decided to improve the synthesis of this compound starting from the method used by Van der Goot in which 1-amino-3-(2-pyridyl)isoquinoline was synthesized from 2-methylbenzonitrile and pyridine-2-carbonitrile (Scheme I).

Scheme I.



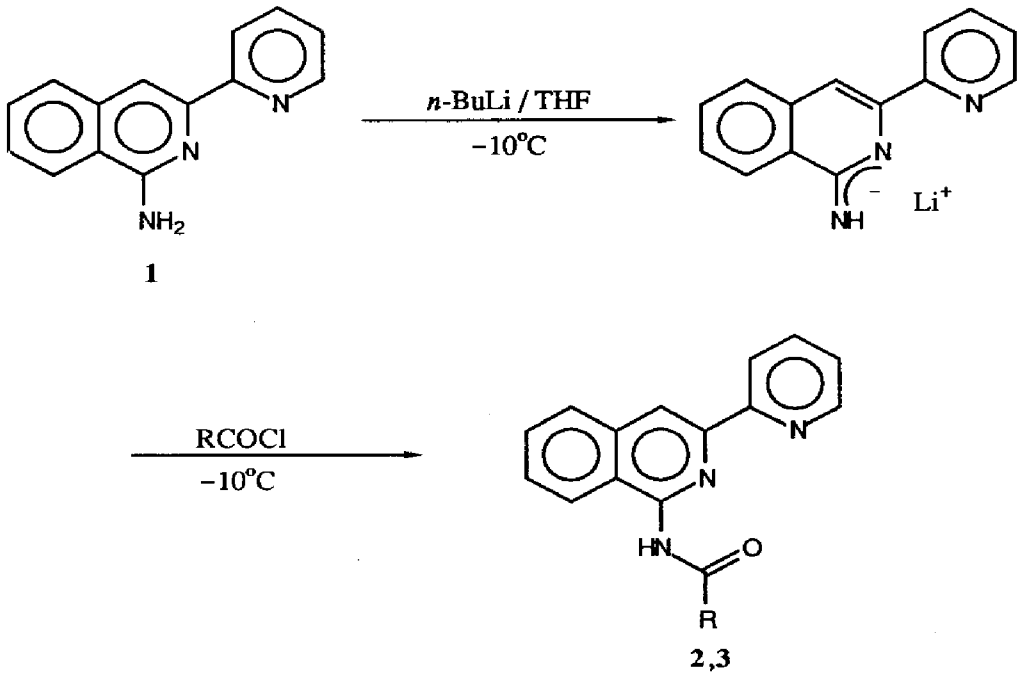
1

Potassium amide is prepared *in situ* by adding potassium at -33°C to liquid ammonia, to which some crystals of ferric nitrate had been added. When the formation of potassium amide is complete the mixture is cooled to -78°C and an equimolar quantity of 2-methylbenzonitrile in anhydrous diethyl ether is added, while keeping the temperature at -78°C. Subsequently an equimolar quantity of pyridine-2-carbonitrile in anhydrous THF is added at that temperature.Then stirring is continued at room temperature to evaporate the ammonia. According to this procedure the yield of 1-amino-3-(2-pyridyl)isoquinoline was increased to 60%.

For the preparation of amides (**2a-f,3a-m**) from acyl chlorides and 1-amino-3-(2-pyridyl) isoquinoline (scheme II) we decided to abstract one of the amine protons first to increase the

nucleophilic power of the amino nitrogen atom. The reaction of this anion with acyl chlorides is then carried out at -10°C. Application of higher reaction temperatures resulted in increasing amounts of diacylated products. By use of this method these amides could be obtained, however, in moderate yields (10-60%). This is probably due to delocalization of the negative charge of the anion of 1-amino-3-(2-pyridyl) isoquinoline (scheme II).

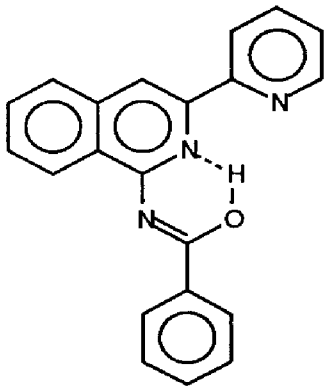
Scheme II.



1

2,3

N-[3-(2-pyridyl)isoquinolin-1-yl]acetamide (**2a**) was not only prepared as has been described above but also by refluxing 40 mmol 1-amino-3-(2-pyridyl)isoquinoline for one hour in 100 mL acetic acid/acetic anhydride (1:1 v/v). However this procedure resulted in poor yields of **2a** and high amounts of diacylated product were obtained.



4

The structure of the synthesized compounds was established by IR, NMR and mass spectrometry. It was remarkable that in contrast with aliphatic amides, aromatic amides except for **3k** are for the most part present in the iminol form (**4**), when dissolved in chloroform. This can be concluded from the NMR spectra, which show an absorption at low field (~16.3 ppm) which can be assigned to a proton participating in an intramolecular hydrogen bond. This iminol form is favoured by conjugation of the phenyl group with the isoquinoline moiety. This may also account for the relatively low predominance of the iminol form in the case of these aliphatic amides. IR spectrophotometry (KBr platelets) revealed that the aromatic amides except for **3a, 3b, 3i, 3j** and **3k** are obtained after recrystallisation in the iminol form.

However, when IR spectrophotometry was performed with solutions of **3a, 3b, 3i** and **3j** in chloroform, the carbonyl absorption disappeared (**3a, 3b**) or was markedly reduced (**3i, 3j**)(results not shown), indicating once again that these amides are present in the iminol form when dissolved in chloroform. The same features have been described for amides derived from 1-aminoisoquinoline [15-18].

As was expected from NMR data, the carbonyl absorption did not disappear in the case of compound **3k**. This compound is present in the amide form in the solid state as well as in solution. This phenomenon is caused by the presence of two ortho substituents in the benzamide moiety. These substituents force the amide moiety and the phenyl group into perpendicular planes, disrupting the overlap of the π orbitals of the carbonyl moiety and the phenyl group. Consequently conjugation of aromatic units in the iminol form is no longer apparent. Although π overlap of the iminol moiety with the isoquinoline nucleus and hydrogen bonding still contribute to the stabilisation of the iminol tautomer, the loss of conjugation of these two aromatic units may be the reason that the iminol form is not favoured over the amide form in the case of compound **3k**.

Biological activity

All of these compounds have been tested with and without the addition of copper to the medium. Without the addition of copper the copper concentration of Adler medium [19] was less than 3 μM [20]. To determine the antimycoplasmal activity of these compounds in the presence of copper, 40 μM $CuSO_4$ was added to the Adler growth medium. This copper concentration however, is far below the toxic level. The minimal inhibitory concentration (MIC) for copper was established to be 400 μM (Table I).

MIC values for aliphatic and aromatic N-[3-(2-pyridyl)isoquinolin-1-yl]amides (**2a-f, 3a-m**) are presented in Tables II and III, respectively. Without copper none of these compounds were active in the concentration range tested. It was not possible to test these compounds in higher concentrations than indicated because they appeared to be poorly soluble

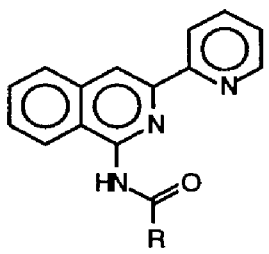
in the growth medium.In the presence of copper, however, all of these amides had about the same antimycoplasmal activity as the parent compound **1**. As a matter of fact under these conditions all compounds except for **2a, 2b, 3e** and **3k** appeared to be somewhat more active than compound **1**.

Table I. MIC values[a] (μM) against *M.gallisepticum* K514 in a modified Adler medium at 37°C.

Compd	without extra copper	extra copper added[b]
$CuSO_4.5H_2O$	400	-
tylosin	0.1	0.1
1	452	0.45

[a] Number of determinations of MIC values is two.[b] 40 μM $CuSO_4$

Table II. MIC values[a] (μM) against *M.gallisepticum* K514 in a modified Adler medium at 37°C.

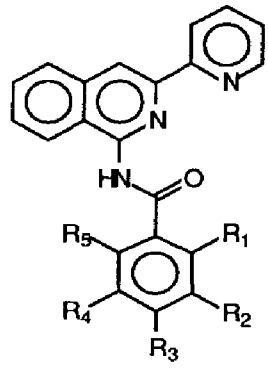


2a-f

Compd	R	without extra copper	extra copper added[b]
2a	CH_3	> 25	1.90
2b	C_2H_5	> 25	0.45
2c	$CH(CH_3)_2$	> 25	0.22
2d	$C(CH_3)_3$	> 25	0.21
2e	$CH(C_2H_5)C_4H_9$	> 170	0.36
2f	C_9H_{19}	> 170	0.34

[a] Number of determinations of MIC values is two. [b] 40 μM $CuSO_4$

Table III. MIC values[a] (μM) against *M.gallisepticum* K514 in a modified Adler medium at 37°C.



3a-m

Compd	R_1	R_2	R_3	R_4	R_5	without extra copper	extra copper added[b]
3a	H	H	H	H	H	12.31	0.10
3b	H	H	CH_3	H	H	>160	0.19
3c	H	H	OCH_3	H	H	>160	0.18
3d	H	H	Cl	H	H	>160	0.35
3e	H	Cl	Cl	H	H	>160	0.64
3f	H	CH_3	H	H	H	> 23	0.09
3g	H	OCH_3	H	H	H	> 23	0.18
3h	H	Cl	H	H	H	> 23	0.35
3i	CH_3	H	CH_3	H	H	>100	0.39
3j	CH_3	H	H	CH_3	H	>100	0.39
3k	CH_3	H	H	H	CH_3	> 25	0.78
3l	H	CH_3	CH_3	H	H	>100	0.39
3m	H	CH_3	H	CH_3	H	>100	0.39

[a] Number of determinations of MIC values is two.[b] 40 μM $CuSO_4$.

Comparison of compounds **2a** and **3a** with the corresponding N-[2-(2-pyridyl)quinazolin-4-yl]amides [8] revealed that they are 20 and 5 times less active respectively. The activity sequence for the aliphatic amides (**2a-f**) is as follows:

$$CH_3 < C_2H_5 < CH(CH_3)_2,\ C(CH_3)_3 > CH(C_2H_5)C_4H_9,\ C_9H_{19}$$

Antimycoplasmal activity increases with chain size from the acetamide derivative (**2a**) up to the t-butylamide (**2d**). A further elongation of the aliphatic chain results in a decrease of antimycoplasmal activity. In the case of aromatic amides (**3a-m**) activity increases in the

following order:

2,6-$(CH_3)_2$ < 3,4-$(Cl)_2$ < other $(CH_3)_2$, 3-Cl, 4-Cl < 4-CH_3, 3-OCH_3, 4-OCH_3 < 3-CH_3, H.

In the presence of copper, compounds **3a** and **3f** are as active as the reference compound tylosin.

Structure-activity relationships

Antimycoplasmal activity is copper dependent for a variety of compounds containing a 2,2'-bipyridyl moiety. Without the addition of a small amount of copper all compounds of the present series except for **3a** are at least more than 200 times less active against *M. gallisepticum* than the reference compound tylosin.

However, upon addition of a little copper all compounds are fairly active against *M.gallisepticum*, the most active compounds being as active as tylosin. Due to this remarkable copper effect it is very likely that these compounds act via their copper complexes as do other 2,2'- bipyridyl analogues [4].

Comparison of the antimycoplasmal activity of these compounds with the activity of the parent compound 1-amino-3-(2-pyridyl)isoquinoline shows that most of the amides are more active. This is probably due to the presence of a third coordination site for the copper atom. It is known that 2,2'-bipyridyl compounds are able to chelate copper very well and that both nitrogen atoms play an important role in the formation of such copper complexes. However, when apart from the bipyridyl nitrogen atoms, the oxygen atom of the amide moiety is involved in the complex formation, the positive charge is more shielded, which might favor the transportation of these complexes across the lipophilic cell membrane.

In a qualitative consideration of a possible structure-activity relationship we only took into account the influence of the part of the molecule which is varied within these series viz. the amide residue, regarding the influence of the 3-(2-pyridyl)isoquinoline part to be constant. When we consider the activity sequence of these aliphatic amides **2a-d**, an increase of activity is paralleled by an increase in lipophilicity of the aliphatic chain. However, a further increase in lipophilicity **(2e, f)** results in a decrease in antimycoplasmal activity. The influence of the lipophilicity of the amide residue is even more pronounced in the case of the aromatic amides. Here an increase in lipophilicity of the aromatic nucleus parallels a decrease in antimycoplasmal activity.

Furthermore, it is obvious from these data that the position of the substituent in the aromatic nucleus has no influence on the biological activity. This also supports the hypothesis that the lipophilicity of the acyl residue is the predominant parameter that determines the antimycoplasmal activity and that other substituent contributions such as electronic features only play a minor role. Also according to Topliss[9], this activity sequence suggests a dependency

on lipophilicity. When we combine results of both aliphatic and aromatic amides, this qualitative approach to a structure-activity relationship suggests the existence of an optimal lipophilicity for antimycoplasmal activity of these amides.

Table IV. Hydrophobic fragmental values of -NHCOR fragments.[a]

Compd	R	Σ_f	MIC(μM)$_{calcd}$[b]	MIC(μM)$_{obsd}$
2a	CH_3	-1.745	1.47	1.90
2b	C_2H_5	-1.226	0.56	0.45
2c	*i*-C_3H_7	-0.707	0.28	0.22
2d	*t*-C_4H_9	-0.188	0.19	0.21
2e	C_7H_{15}	1.369	0.30	0.36
2f	C_9H_{19}	2.407	1.68	0.34
3a	C_6H_5	0.550	0.17	0.10
3g	3-$OCH_3(C_6H_4)$	0.630	0.18	0.18
3c	4-$OCH_3(C_6H_4)$	0.630	0.18	0.18
3f	3-$CH_3(C_6H_4)$	1.069	0.23	0.09
3b	4-$CH_3(C_6H_4)$	1.069	0.23	0.19
3h	3-$Cl(C_6H_4)$	1.292	0.28	0.35
3d	4-$Cl(C_6H_4)$	1.292	0.28	0.35
3e	3,4-$(Cl)_2(C_6H_3)$	2.034	0.80	0.64
3i	2,4-$(CH_3)_2(C_6H_3)$	1.588	0.40	0.39
3j	2,5-$(CH_3)_2(C_6H_3)$	1.588	0.40	0.39
3l	3,4-$(CH_3)_2(C_6H_3)$	1.588	0.40	0.39
3m	3,5-$(CH_3)_2(C_6H_3)$	1.588	0.40	0.39

[a]see reference 21.[b] calculated from equation 2.

The existence of an optimal lipophilicity for antimycoplasmal activity is supported by results of a quantitative approach to a structure-activity relationship. As for the qualitative approach, we only took into account the influence of the alkyl or aryl amide moiety. Hydrophobic fragmental values (Σ_f) were calculated for the -NHCOR residue of these compounds according to Rekker [21]. Since lipophilicity is increased through conjugation in the iminol tautomer for aromatic amides only, 1x c_m (magic constant = 0.289) is added to the hydrophobic fragmental values of these aromatic amides (Table IV). By multiple regression analysis the following equation is obtained :

$$-\log MIC = 6.720\ (\pm\ 0.075) + 0.159\ (\pm\ 0.047)\ \Sigma_f - 0.178\ (\pm\ 0.036)\ (\Sigma_f)^2 \qquad (1)$$

n = 18 r = 0.797 s = 0.193 F = 13.092

When compounds **2f**, **3a** and **3f** are omitted for legitimate statistical reasons (residual > 2x standard deviation) a much better equation is obtained:

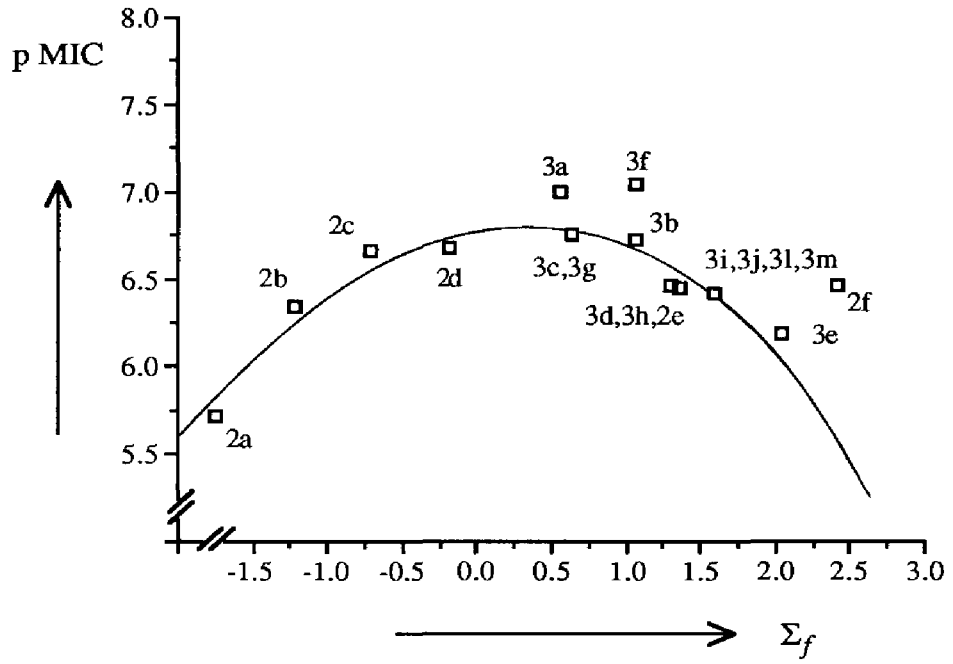
$$-\log MIC = 6.754\ (\pm\ 0.039) + 0.135\ (\pm\ 0.020)\ \Sigma_f - 0.225\ (\pm\ 0.020)(\Sigma_f)^2 \qquad (2)$$

n = 15 r = 0.959 s = 0.080 F = 69.229

So, for fifteen of the original series of eighteen compounds a very good correlation is found between antimycoplasmal activity and lipophilicity.

According to equation 2 the dependency of the biological parameter on hydrophobic fragmental values is parabolic in nature, indicating that an optimal lipophilicity for antimycoplasmal activity exists (Figure 1). From this equation, the optimal lipophilicity for the amide residue is determined as $\Sigma_f = 0.30$.

Figure I. p MIC vs Σ_f. For the meaning of 2a-3l, see Table II and III.



However, the influence of compound **2a** on the result of the regression analysis is considerable and the shape of the maximum is rather flat. The same feature was found as a

result of a quantitative structure-activity relationship study concerning antimycoplasmal activity of compounds containing an ortho-substituted 2,2'-bipyridyl moiety [22].

It should be noted that compound **3k** is left out of these regression analyses deliberately. As explained above, this dimethyl-substituted benzamide in contrast to all other dimethyl-substituted benzamides, is present in the amide form and because of this it is less lipophilic. So, according to the equation obtained from multiple regression analysis, one would expect this compound to be somewhat more active than the other dimethyl-substituted benzamides. However, compound **3k** is less active. Due to this anomalous behaviour and to the different structure of compound **3k**, we apparently have to consider this compound as belonging to a different series of compounds.

Conclusions

It can be concluded from this study that although amides (**2a-f,3a-m**) derived from 1-amino-3-(2-pyridyl)isoquinoline are not active themselves, they are very potent antimycoplasmal agents in the presence of a small amount of copper. Therefore, it is very likely that copper complexes of these compounds are involved in the growth inhibiting process.

Acylation of 1-amino-3-(2-pyridyl)isoquinoline (**1**) resulted in the formation of the most potent derivatives having a five fold increase in antimycoplasmal activity. The potency of the most active compounds, e.g. **3a**, **3f**, is comparable to the therapeutically useful anti-mycoplasmal drug tylosin.

Furthermore, it is established that the antimycoplasmal activity is apparently dependent on the hydrophobic fragmental value of the amide residue. A quantitative approach to a structure-activity relationship revealed a good correlation between antimycoplasmal activity and lipophilicity parameters as Σ_f and $(\Sigma_f)^2$. The optimal lipophilicity of the amide residue of N-[3-(2-pyridyl) isoquinolin-1-yl]amides regarding antimycoplasmal activity was calculated as $\Sigma_f = 0.30$.

Experimental Section

Chemistry

All starting materials were commercially available and of the highest purity obtainable. Acyl chlorides were prepared from the corresponding carboxylic acids by standard methods [11,12] and distilled prior to use in the acylation reaction. Melting points were determined using a Mettler FP5/FP52 apparatus. NMR spectra were recorded on a Bruker WH-90 90 MHz spectrophotometer at 21°C. Chemical shifts are expressed in ppm relative to tetramethylsilane. Infrared spectra were recorded on a Jasco IRA II spectrophotometer. Recording of mass spectra

and peak matching were performed with a Varian CH 5 DI mass spectrometer, electron impact 70 eV. Analytical results for compounds indicated by the molecular formula were within ± 0.4 % of the theoretical values.

Synthesis

1-amino-3-(2-pyridyl)isoquinoline(1)

In a thoroughly dried three-necked flask equipped with a mechanical stirrer, 0.5 mol potassium amide is freshly prepared in 500 mL ammonia. The mixture is then cooled to -78°C. Subsequently 0.5 mol 2-methylbenzonitrile in 200 mL anhydrous diethyl ether is added slowly while keeping the temperature at -78°C. When the addition is complete, a solution of 0.5 mol 2-cyanopyridine in 250 mL anhydrous THF is run into the flask within twenty minutes. The cooling device is then removed, and stirring is continued overnight to evaporate the ammonia. When necessary, a small amount of anhydrous THF is added, and the mixture is refluxed for two hours. The mixture is cooled to room temperature and hydrolyzed by the addition of a small amount of water. After evaporation of the organic phase diethyl ether is
poured on the remaining mixture. Most of the 1-amino-3-(2-pyridyl)isoquinoline precipitated and was collected by filtration. A minor part could be isolated from the fitrate by extraction with diethyl ether.The product was crystallized from methanol.

Yield 66.3 g (60%) of white platelets, mp 152.0-153.0°C;

NMR ($CDCl_3$) [23]: δ 5.32 (br s, 2H, NH_2), 7.25 (ddd, $J_{3'-5'}$=1.2Hz, $J_{5'-6'}$=4.8Hz, $J_{4'-5'}$=7.4Hz, 1H, H-5'), 7.39-7.87 (m, 5H, H-5, H-6, H-7, H-8, H-4'), 8.16 (d, J_{4-8}=0.5Hz, 1H, H-4), 8.38 (ddd, $J_{3'-6'}$=0.9Hz, $J_{3'-5'}$= 1.4Hz, $J_{3'-4'}$=7.8Hz, 1H, H-3'), 8.71 (ddd, $J_{3'-6'}$=0.9Hz, $J_{4'-6'}$=1.75Hz, $J_{5'-6'}$=4.8Hz, 1H, H-6');

IR(KBr,cm^{-1}): 3300 and 3190(NH_2), 1635(s), 1620(s), 1580(s), 1565(s), 1495(s) (C=C,C=N), 1475(s), 1420, 990, 785(s), 740(s);

MS, m/e 221.0964 (M^+), 221.0953 ($C_{14}H_{11}N_3$).

***General procedure for the synthesis of amides from 1-amino-3-(2-pyridyl)isoquinoline* (2,3)**

A solution of 0.02 mol 1-amino-3-(2-pyridyl)isoquinoline (**1**) in 40 mL anhydrous THF was stirred under a nitrogen atmosphere and cooled to -10°C. Subsequently 12.5 mL 1.6M *n*-butyllithium in hexane was added dropwise and stirring was continued for ten minutes. Then, 0.02 mol of freshly distilled acyl chloride in 5 mL anhydrous THF was added and while keeping the reaction mixture at -10°C, stirring was continued for 1 hour. The ice bath was removed and when the mixture had reached room temperature it was hydrolyzed with water.
The reaction mixture was extracted with chloroform. The combined chloroform layers were washed with a dilute sodium bicarbonate solution, dried with anhydrous potassium carbonate and, after filtration, evaporated to dryness.

N-[3-(2-pyridyl)isoquinolin-1-yl]acetamide (2a)

The crude product was crystallized from $CHCl_3$.The solid material was filtered and washed with a little diethyl ether to remove traces of 1-amino-3-(2-pyridyl)isoquinoline (**1**). After this purification step, the residue was crystallized from $CHCl_3$, yielding 2.76 g (53%) of very small , white needles, mp 186.0-187.4°C;

NMR ($CDCl_3$): δ 2.58 (s, 3H, CH_3), 7.33 (ddd, J=7.6/4.8/1.4 Hz,1H, H-5'), 7.51-8.07 (m, 5H),8.24 (br s, 0.4H, NH), 8.38 (d, J=7.2 Hz, 1H, H -3'), 8.62 (s,1 H, H-4), 8.73 (d, J=4.5 Hz, 1H, H-6');

IR (KBr,cm^{-1}): 3200, 3100(NH), 2960(CH), 1680(C=O), 1625, 1570(C=C,C=N), 1500(NH), 1470(CH), 1425, 1375(s), 1330(s), 785, 750, 730, 670;

MS, m/e 263.1057 (M^+), 263.1058 ($C_{16}H_{13}N_3O$). Anal. ($C_{16}H_{13}N_3O$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl]propionamide (2b)

The crude product was crystallized from $CHCl_3$. The precipitate was recrystallized from $CHCl_3$ to remove trace amounts of 1-amino-3-(2-pyridyl)isoquinoline (**1**).

Yield 2.72 g (49%) of small white needles, mp 194.5-196.0°C;

NMR ($CDCl_3$): δ 1.35 (t, J=7.2 Hz, 3H, CH_3), 2.90 (q, J=7.2 Hz, 2H, CH_2), 7.32 (ddd, J=7.4/4.5/1.0 Hz, 1H, H-5'), 7.52-8.05 (m, 5H), 8.25 (br s, 0.4H, NH), 8.38 (d, J=7.6 Hz, 1H, H-3'), 8.65 (s, 1H, H-4'), 8.74 (d, J=4.5 Hz, 1H, H-6');

IR (KBr,cm^{-1}): 3180, 3080(NH), 2960(CH), 1680(C=O), 1620(s), 1580(s), 1570(s)(C=C,C=N), 1500(s)(NH), 1470(CH), 1420(s), 1390, 1330(s), 1290, 1270, 780, 750, 730;

MS m/e 277.1230 (M^+), 277.1215 ($C_{17}H_{15}N_3O$). Anal. ($C_{17}H_{15}N_3O$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl]-2-methylpropionamide(2c)

This product was obtained as small white needles in the same way as **2b**.

Yield 2.74 g (47%), mp 194.2-196.3°C;

NMR ($CDCl_3$): δ 1.38 (d, J=7.2 Hz, 6H, CH_3), 3.02 (m, 1H, CH), 7.31 (ddd, J=7.2/4.5/1.1 Hz, 1H, H-5'), 7.49-8.0 (m, 5H), 8.11 (br s, 0.5H, NH), 8.36 (d, 7.6 Hz, 1H, H-3'), 8.63 (s, 1H, H-4), 8.72 (d, J=4.5 Hz, 1H, H-6');

IR (KBr,cm^{-1}): 3250(NH), 2960(CH), 1665(C=O), 1625, 1580(s), 1570(s), 1510, 1490 (C=C,C=N), 1425, 1400(s), 1340(s), 1300, 1210, 1090, 790, 780, 740;

MS, m/e 291.1380 (M^+), 291.1371 ($C_{18}H_{17}N_3O$). Anal. ($C_{18}H_{17}N_3O$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl]-2,2-dimethylpropionamide (2d)

The crude reaction mixture was dissolved in chloroform and subsequently extracted several times with a buffer solution, pH = 5.0. The chloroform layer was dried with anhydrous potassium carbonate and, after filtration, evaporated to dryness. The residue was dissolved in dry diethyl ether. Diethyl ether saturated with hydrochloric acid was added dropwise to this solution. Initially, a small amount of the hydrochloride salt of

1-amino-3-(2-pyridyl)isoquinoline precipitated. The mixture was filtrated, and the filtrate was further acidified as described above. Eventually, the hydrochloride salt of **2d** was isolated by filtration of the acidified mixture and crystallized from ethanol.

Yield 2.12 g (31%), mp 156.6 - 158.3 °C, mp free base 56.0 - 58.3 °C [24];

NMR (DMSO-d6): δ 1.40 (s, 9H, CH_3), 7.65-8.39 (m, 6.5 H), 8.61 (d, J =7.8 HZ, 1H, H-3'), 8.85 (dd, J = 4.5 Hz, 1H, H-6'), 8.93 (s, 1H, H-8), 10.32 (br s, 0.5 H, NH);

IR (KBr,cm^{-1})(free base): 3280(br), 3050(NH), 2960(CH), 1665(C=O), 1620, 1580, 1535 (C=C,C=N), 1475, 1425, 1390, 1340, 1140, 790, 780, 740;

MS, 305.1520 (M^+), 305.1528 ($C_{19}H_{19}N_3O$). Anal. ($C_{19}H_{19}N_3O$) H, N; C: calcd, 74.73; found, 73.86.

N-[3-(2-pyridyl)isoquinolin-1-yl]-2-ethylhexanamide (2e)

The crude reaction product is crystallized several times from methanol, yielding 4.08 g (59%) of white needles, mp 177.3-178.8°C;

NMR ($CDCl_3$): δ 0.79-1.95 (m, 14H, C_2H_5, C_4H_9), 2.43-2.83 (m, 1H, CH), 7.32 (dd, J=7.6/4.7 Hz, 1H, H-5'), 7.53-8.21 (m, 5.5H), 8.42 (d, J=7.6 Hz, 1H, H-3'), 8.66 (s, 1H, H-4'), 8.76 (d, J=4.5 Hz, 1H, H-6');

IR (KBr,cm^{-1}): 3240(NH), 2960, 2920(CH), 1665(C=O), 1625, 1580, 1560, 1520 (C=C,C=N), 1425, 1380, 1340, 1215, 890, 790(s), 780, 750, 740, 670, 620;

MS, m/e 347.1988 (M^+), 347.1997 ($C_{22}H_{25}N_3O$). Anal. ($C_{22}H_{25}N_3O$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl]decanamide (2f)

This product was obtained in the same way as **2e**.

Yield 3.75 g (50%), mp 122.4 -124.5°C;

NMR ($CDCl_3$): δ 0.80-1.98 (m, 17H, C_8H_{17}), 2.83 (t, J=7.2 Hz, 2H, CH_2), 7.33 (ddd, J=7.6/4.5/1.1 Hz, 1H, H-5'), 7.55-8.14 (m, 5.5H), 8.34 (d, J=7.2 Hz, 1H, H-3'), 8.59 (s, 1H, H-4), 8.77 (d, J=4.5 Hz, 1H, H-6');

IR (KBr,cm^{-1}): 3240(NH), 2920, 2840(CH), 1665(C=O), 1625, 1580, 1520(C=C,C=N), 1190, 890, 790, 740, 670;

MS, m/e 375.2321 (M^+), 375.2310 ($C_{24}H_{29}N_3O$). Anal. ($C_{24}H_{29}N_3O$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl]benzamide (3a)

This product was obtained by crystallization of the crude product from CH_3OH / $CH_3COOC_2H_5$.

Yield 2.80 g (43%), mp 189.9-191.0°C;

NMR ($CDCl_3$): δ 7.26-8.12 (m, 10H), 8.55 (m, 2H), 8.87 (d, J=4.2 Hz, 1H, H-6'), 9.01 (d, J=7.5 Hz, 1H, H-3'), 16.30 (br s, 0.6H,OH);

IR (KBr,cm^{-1}): 3240 (NH), 3040(CH), 1660(C=O), 1620, 1575, 1520(C=C,C=N), 1470, 1420, 1390, 1335, 1300, 1145, 900, 885, 790, 780, 740, 720;

MS, m/e 325.1212 (M^+), 325.1215 ($C_{21}H_{15}N_3O$). Anal. ($C_{21}H_{15}N_3O$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl]-4-methylbenzamide (3b)

The crude product was crystallized from methanol and the resulting precipitate was purified via column chromatography using silicagel 60 H with diethyl ether as eluent.

Yield 3.1 g (46%), mp 192.5-194.0°C;

NMR ($CDCl_3$): δ 2.43 (s, 3H, CH_3), 7.28 and 8.42 (AA'BB' system, J_{ab}=8.1 Hz, 4H), 7.24-7.42 (m, 1H, H-5'), 7.54-8.03 (m, 6.4H), 8.86 (d, J=4.5 Hz, 1H, H-6'), 9.05 (d, J=7.6 Hz, 1H, H-3'), 16.33 (br s, 0.6H, OH);

IR (KBr,cm^{-1}): 3240(NH), 3040(CH), 1655(C=O), 1625, 1610, 1575(s), 1525 (C=C,C=N), 1490, 1470, 1400, 1340, 1300, 1145, 790, 745;

MS, m/e 339.1375(M^+), 339.1372 ($C_{22}H_{17}N_3O$). Anal. ($C_{22}H_{17}N_3O$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl]-4-methoxybenzamide (3c)

The crude product was crystallized several times from $CH_3OH/CH_3COOC_2H_5$, yielding 3.69 g (52%) of the amide, mp 161.4-162.9°C;

NMR ($CDCl_3$): δ 3.91 (s, 3H, OCH_3), 6.97 and 8.49 (AA'BB' system, J_{ab}=8.4 Hz, 4H), 7.49-8.07 (m, 7.2H), 8.86 (d, J=4.5 Hz, 1H, H-6'), 9.01 (d, J=7.6 Hz, 1H, H-3'),16.26 (br s, 0.6H, OH);

IR (KBr,cm^{-1}): 3400(OH), 3040(CH), 1620, 1570(s), 1550(s), 1520(s)(C=C,C=N), 1465(s), 1410, 1390, 1370, 1320(s), 1245(s), 1140(s), 1100, 850, 775(s), 755, 725, 690;

MS, m/e 355.1320 (M^+), 355.1320 ($C_{22}H_{17}N_3O_2$). Anal. ($C_{22}H_{17}N_3O_2$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl]-4-chlorobenzamide (3d)

The crude reaction mixture was crystallized several times from $CH_3OH/CH_3COOC_2H_5$, yielding 3.8 g (53%) of the amide, mp 166.2-167.4°C.

NMR ($CDCl_3$): δ 7.42 and 8.45 (AA'BB' system, J_{ab}=8.3 Hz, 4H), 7.26-8.08 (m, 8H), 8.87(d, J =4.5 Hz, 1H, H-6'), 9.01 (d, J=7.6 Hz, 1H, H-3'), 16.23 (br s, 0.6H, OH);

IR (KBr,cm^{-1}): 3400(OH), 3040(CH), 1610, 1575, 1525(C=C,C=N), 1465, 1390, 1325, 1240, 1140, 1080, 1010, 880, 850, 780, 760, 740, 690;

MS, m/e 359.0807 (M^+), 359.0825 ($C_{21}H_{14}{}^{35}ClN_3O$). Anal. ($C_{21}H_{14}ClN_3O$) C, H, N; Cl: calcd 19.85; found 9.43.

N-[3-(2-pyridyl)isoquinolin-1-yl]-3,4-dichlorobenzamide (3e)

The crude product was crystallized from acetone,yielding 2.36 g (30%) of very small, light-yellow needles, mp 207.4-208.7°C;

NMR ($CDCl_3$): δ 7.37 (ddd, J=7.2/4.7/1.3 Hz, 1H, H-5'), 7.54 (d, J=8.1 Hz, 1H, Phe H-5), 7.61-8.09 (m, 7H), 8.33 (dd, J=8.1/1.8 Hz, 1H, Phe H-6), 8.61 (d, J=1.8 Hz, 1H, Phe H-2), 8.88 (d, J=4.7 Hz, 1H, H-6'), 8.99 (d, J=7.2 Hz, 1H, H-3'), 16.23 (br s, 0.7H, OH);

IR (KBr,cm^{-1}): 3040(CH), 1630, 1580, 1550, 1525(C=C,C=N), 1470, 1440, 1375, 1325, 1140, 900, 840,

780(s), 760, 740, 730, 670;

MS, m/e 393.0456 (M^+), 393.0435 ($C_{21}H_{13}{}^{35}Cl_2N_3O$). Anal. ($C_{21}H_{13}Cl_2N_3O$) C, H, N, Cl.

N-[3-(2-pyridyl)isoquinolin-1-yl]-3-methylbenzamide (3f)

The crude product was first crystallized from $CH_3OH/CH_3COOC_2H_5$. The precipitate was purified via column chromatography using silicagel 60 H with diethyl ether as eluent.The fractions containing **3f** were pooled and after evaporation of the solvent the product was crystallized from $CH_3OH/CH_3COOC_2H_5$.

Yield 2.78 g (41%), mp 161.2-162.4°C;

NMR ($CDCl_3$): δ 2.49 (s, 3H, CH_3), 7.27-8.13 (m, 9.6H), 8.34 (m, 2H), 8.87 (d, J=4.5 Hz, 1H, H-6'), 9.05 (d, J=7.2 Hz, 1H, H-3'), 16.32 (br s, 0.6H, OH);

IR (KBr,cm^{-1}): 3400(OH), 3040(CH), 1625, 1575(s), 1550(s), 1520(C=C,C=N), 1470, 1440, 1390, 1315(s), 1240, 1140, 780, 740, 730;

MS, m/e 339.1345 (M^+), 339.1372 ($C_{22}H_{17}N_3O$). Anal. ($C_{22}H_{17}N_3O$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl]-3-methoxybenzamide (3g)

The crude product is crystallized several times from $CH_3OH/CH_3COOC_2H_5$,yielding 2.56 g (36%) of the amide, mp 159.4-160.8°C;

NMR ($CDCl_3$): δ 3.93 (s, 3H, OCH_3), 7.03-8.21 (m, 11.4H), 8.85 (d, J=4.5 Hz, H-6'), 9.01 (d, J=7.5 Hz, 1H, H-3'), 16.28 (br s, 0.6H, OH);

IR (KBr,cm^{-1}): 3400(OH), 3040(CH), 1625, 1575(s), 1530(s)(C=C,C=N), 1465, 1450, 1390, 1320(s), 1270, 1240, 1140, 1040, 830, 775, 755, 745, 730, 670;

MS, m/e 355.1290 (M^+), 355.1320 ($C_{22}H_{17}N_3O_2$). Anal. ($C_{22}H_{17}N_3O_2$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl]-3-chlorobenzamide (3h)

The crude product was crystallized twice from $CH_3OH/CH_3COOC_2H_5$, yielding 2.75 g (38%) of the amide, mp 201.1-201.9°C;

NMR ($CDCl_3$): δ 7.26-8.01 (m, 9.3H), 8.39 (dt, J=7.2/2.1 Hz, 1H, Phe H-6), 8.53 (s, 1H, Phe H-2), 8.86 (d, J=4.5 Hz, 1H,H-6'), 8.99 (d, J=7.2 Hz, 1H, H-3'),16.23 (br s, 0.7H, OH);

IR (KBr,cm^{-1}): 3400(OH), 3040(CH), 1625, 1570(s), 1545, 1520(C=C,C=N),1465, 1390, 1320, 1245, 1140, 1065, 1045, 890, 810, 780, 750, 730, 665;

MS, m/e 359.0798 (M^+), 359.0825 ($C_{21}H_{14}N_3{}^{35}ClO$). Anal. ($C_{21}H_{14}N_3ClO$) C, H, N, Cl.

N-[3-(2-pyridyl)isoquinolin-1-yl]-2,4-dimethylbenzamide (3i)

After crystallization of the crude product from $CH_3OH/CH_3COOC_2H_5$, the compound was recrystallized from acetone.Yield 0.78 g (11%), mp 172.6-173.5 °C;

NMR ($CDCl_3$): δ 2.40 (s, 3H, 4-CH_3), 2.56 (s, 1.7 H, 2-CH_3), 2.80 (s, 1.3 H, 2-CH_3), 7.10 (br s, 2 H, Phe

H-3, Phe H-5), 7.20-7.45 (m, 1H, H-5'), 7.53-8.50 (m, 7.7 H), 8.60-9.04 (m, 2H, H-3', H-6'), 16.18 (br s, 0.3 H, OH).

IR (KBr,cm^{-1}): 3400 (br) (OH), 3240 (s) (OH), 3000 (CH), 1660 (s) (C=O), 1635, 1610, 1580, 1510 (C=C,C=N), 1475, 1425, 1400, 1330, 1300, 1255, 1230,1170, 1140, 885, 835, 790, 770, 740, 690, 670.

MS, m/e 353.1531(M^+), 353.1528 ($C_{23}H_{19}N_3O$). Anal. ($C_{23}H_{19}N_3O$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl]-2,5-dimethylbenzamide (3j)

This product was obtained in the same way as compound **3i**.

Yield 0.92 g (13%), mp 186.1-187.4°C;

NMR ($CDCl_3$): δ 2.38 (s, 3H, 5-CH_3), 2.52 (s, 1.8H, 2-CH_3),2.76 (s, 1.2H, 2-CH_3), 7.20 (br s, 2H, Phe H-3, Phe H-4), 7.26-7.45 (m, 1H, H-5'), 7.59-8.35 (m, 7.7 H), 8.68-9.02 (m, 2H, H-3', H-6'), 16.20 (br s, 0.3 H, OH).

IR (KBr, cm^{-1}): 3400 (br) (OH), 3260 (s) (OH), 3000 (CH), 1660 (s) (C=O), 1625 (w), 1580, 1560, 1510, (C=C,C=N), 1485, 1425, 1400, 1340, 1300, 1280, 1200, 990, 940, 920, 900, 860, 840, 810, 790, 740, 675.

MS, m/e 353.1531 (M^+), 353.1528 ($C_{23}H_{19}N_3O$). Anal. ($C_{23}H_{19}N_3O$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl]-2,6-dimethylbenzamide (3k)

The crude product was crystallized from CH_3OH. The precipitate appeared to be the disubstituted product. This procedure was repeated and after evaporation of the CH_3OH from the filtrate, compound **3k** was obtained after crystallization from $CH_3OH/CH_3COOC_2H_5$.

Yield 0.64 g (9%) mp 231.4-232.4°C;

NMR ($CDCl_3$): δ 2.43 (s, 6H, CH_3), 7.06-7.35 (m, 4H, H-5', Phe H-3, Phe H-4, Phe H-5), 7.60-8.25 (m, 7H), 8.59-8.92 (m, 2H);

IR (KBr, cm^{-1}): 3170 (NH), 3040 (CH), 2920 (CH_3), 1640 (s) (C=O), 1565, 1500 (C=C,C=N), 1465, 1420, 1370, 1340, 1320, 1280, 1270, 1245, 1130, 1070, 990, 950, 880, 810, 755, 730, 710, 670.

MS, m/e 353.1534 (M^+), 353.1528 ($C_{23}H_{19}N_3O$). Anal. ($C_{23}H_{19}N_3O$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl]-3,4-dimethylbenzamide (3l)

This product was obtained in the same way as compound **3i**.

Yield 2.54 g (36%) mp 177.8-178.6°C;

NMR ($CDCl_3$): δ 2.35 (s, 3H, 3-CH_3), 2.37 (s, 3H, 4-CH_3), 7.16-7.40 (m, 2H, H-5', Phe H-5), 7.50-8.06 (m, 6H), 8.20-8.33 (d, 2H, Phe H-6, Phe H-2), 8.61 (br s, 0.5H, NH), 8.86 (d, J=4.5 Hz, 1H, H-6'), 9.03 (d, J=7.0 Hz, 1H, H-3'), 16.24 (br s, 0.5H, OH).

IR (KBr, cm^{-1}): 3400 (br) (OH), 3050 (CH), 1630 (w), 1575, 1525 (C=C,C=N), 1470,1440, 1400, 1370, 1315, 1150, 1100, 900, 840, 785, 765, 735.

MS, m/e 353.1534 (M^+), 353.1528 ($C_{23}H_{19}N_3O$). Anal. ($C_{23}H_{19}N_3O$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl]-3,5-dimethylbenzamide (3m)

This product was obtained in the same way as compound **3i**.

Yield 2.90 g (41%) mp 188.6-189.4°C;

NMR ($CDCl_3$): δ 2.40 (s, 6H, CH_3), 7.18 (br s, 1H, Phe H-4), 7.24-7.40 (m, 1H, H-5'), 7.53-8.36 (m, 5H), 8.20 (br s, 2H, Phe H-2, Phe H-6), 8.60 (br s, 0.3 H, NH), 8.82 (d, J=4.2 Hz, 1H, H-6'), 8.98 (d, J=7.5 Hz, 1H, H-3'), 16.22 (br s, 0.7 H, OH);

IR (KBr, cm^{-1}): 3400 (br) (OH), 3040 (CH), 1630 (w), 1575, 1545, 1520 (C=C,C=N), 1465, 1450, 1440, 1390, 1370, 1320, 1270, 1240, 1140, 1115, 1080, 870, 850, 820, 785, 775, 760, 740, 730, 670.

MS, m/e 353.1520 (M^+), 353.1528 ($C_{23}H_{19}N_3O$). Anal. ($C_{23}H_{19}N_3O$) C, H, N.

Biological activity

Nutrient medium

All experiments with *Mycoplasma gallisepticum* were done in a growth medium which was a modification of the medium used by Adler [25] to cultivate this microorganism. This modified Adler medium contained 14.8 g bacteriological peptone, 5.0 g yeast extract powder, 8.16 g D-glucose.H_2O, 3.7 g NaCl, 1.79 g $Na_2HPO_4.2H_2O$, 21 mg phenol red (pH range 6.8 - 8.4), 150 mL heat-inactivated (56°C for 30 minutes) horse serum and 10^6 IU benzylpenicillin G per liter final medium.

The medium components were dissolved in twice-distilled water, and the pH of the solution was adjusted to 8.0 with a concentrated sodium hydroxide solution. Before adding the horse serum and the benzylpenicillin, sterilization was performed by heating at 110°C for 30 minutes.

Materials

Bacteriological peptone and yeast extract powder were purchased from OXOID Limited, Basingstoke, Hampshire, England.

Sterile donor horse serum was obtained from Flow Laboratories, United Kingdom.

Benzylpenicillin G was a generous gift from Gist-brocades N.V., Delft,The Netherlands.

All chemicals used were of the highest obtainable quality.

Apparatus

Optical density of growing cultures were determined at 660 nm using a Zeiss PMQ3 spectrophotometer. pH measurements were performed with a saturated calomel electrode. Test tubes were incubated in a waterbath at 37°C.

Test organism

Mycoplasma gallisepticum K514, kindly supplied by the research management of Gist-brocades N.V., was used as the test organism. *Mycoplasma gallisepticum* strains can be stored at -20°C for several months [26]. After thawing at room temperature the culture was transferred to a bottle with fresh Adler medium in such a way that the original culture was diluted ten times. The culture was incubated overnight at 37°C.

When the pH of the culture had dropped to 6.8 and the density (determined as A_{660nm}) had reached a value of 0.22, the culture was used for inoculation purposes. The remaining part was stored at -20°C.

Determination of antimycoplasmal activity

The antimycoplasmal activity of all compounds was determined in the presence or the absence of copper and expressed as the minimal inhibitory concentration (MIC). In the former case, the final concentration in the test tube was 40 μM $CuSO_4$. Tylosin and compound **1** were included as controls in every test. All compounds were dissolved in dimethylsulfoxide whereas tylosin was dissolved in water. It was established that DMSO in the final concentration in the Adler medium (1.25%) has no effect on Mycoplasmal growth. Serial two fold dilutions (in duplicate) of test compounds were made in Adler medium. Each tube, containing 3 mL of medium, was inoculated with 1 mL of a fresh culture of *Mycoplasma gallisepticum* K514, and these mixtures were incubated at 37°C for 24 hours. Mycoplasmal growth was indicated by a change in color of the indicator present in the medium. The minimal inhibitory concentration was determined as the lowest concentration which did not cause a change in color.

Data processing

Statistical correlations were performed by using a commercial multiple lineair regression program (Statworks™ Cricket Software Inc., Philadelphia, USA).The figures in parentheses are the standard errors of regression coefficients.The parameters included in each equation are significant on a 1% level. For a given equation , n is the number of compounds, r is the multiple correlation coefficient, s is the standard error of estimate and F represents the value of the F-test.

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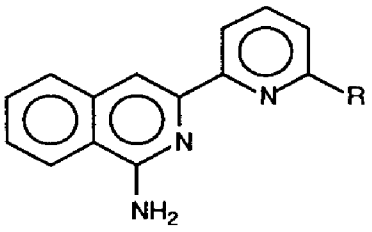
Chapter 4

SYNTHESIS AND COPPER-DEPENDENT ANTIMYCOPLASMAL ACTIVITY OF 1-PHENACYL-3-(2-PYRIDYL)ISOQUINOLINES AND AMIDES DERIVED FROM 1-AMINO-3-(6-METHYL-2-PYRIDYL)ISOQUINOLINE

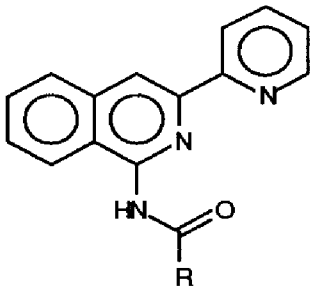
Introduction

In chapter 3 it was shown that acylation of the amino group of 1-amino-3-(2-pyridyl)-isoquinoline (**1**) resulted in more active compounds against *M. gallisepticum*. In this chapter the influence of structural modification of these amides on antimycoplasmal activity is described.

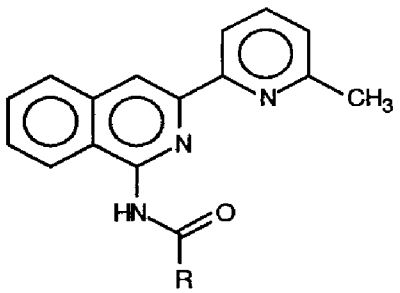
Pijper [1-3] showed for various compounds containing a 2,2'-bipyridyl moiety that antimycoplasmal activity could be enhanced by introduction of substituents at both positions adjacent to the nitrogen atoms of the heterocycle. Because in the case of amides derived from 1-amino-3-(2-pyridyl)isoquinoline one of these positions is still unoccupied, a further increase of antimycoplasmal activity might be obtained by substitution of this position too.



1 R= H
2 R= CH_3



3a R= CH_3
3b R= C_6H_5
3c R= 4-$OCH_3(C_6H_4)$
3d R= 4-$Cl(C_6H_4)$
3e R= 3,4-$Cl_2(C_6H_3)$

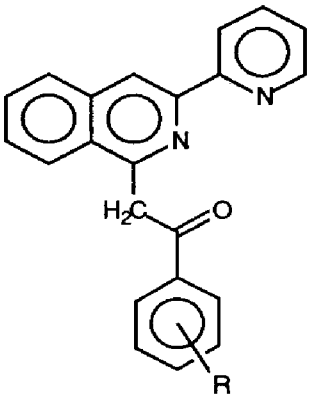


4a R= CH_3
4b R= C_6H_5

As the synthesis of 1-amino-3-(6-methyl-2-pyridyl)isoquinoline (**2**) has been described by Pijper *et al.* [1] we used this compound as starting material for the preparation of amides derived thereof (**4a,b**). Comparison of the antimycoplasmal activity of these compounds with the analogous derivatives (**3a,b**) of 1-amino-3-(2-pyridyl)isoquinoline, which have been described in the previous chapter, will provide information about the influence of an additional ortho substituent on this activity.

Increased antimycoplasmal activity of amides derived from 1-amino-3-(2-pyridyl)-

isoquinoline might be a consequence of the amide moiety operating as a third coordination site for copper [4]. In this study we therefore also investigated the influence of structural modification within this part of the molecule. So, 1-phenacyl-3-(2-pyridyl)isoquinolines **5a-d** are synthesized from 1-chloro-3-(2-pyridyl)isoquinoline and the respective acetophenones.



5a R= H
5b R= 4-OCH_3
5c R= 4-Cl
5d R= 3,4-Cl_2

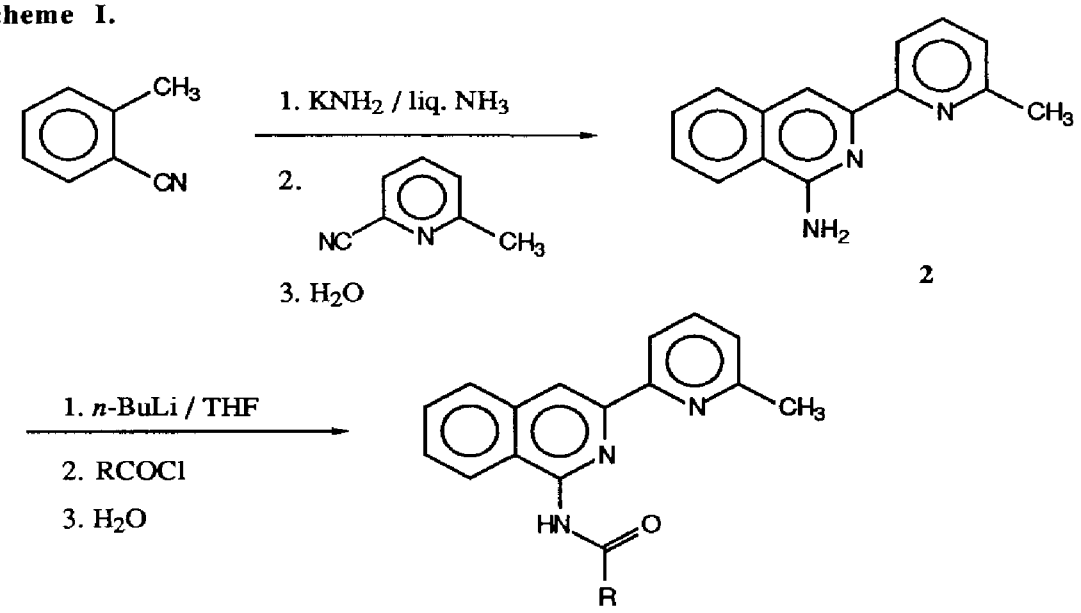
Comparison of the structure of these compounds **5** with amides **3** shows that in fact the amide function is changed into a ketone function in which the oxygen atom might be still available for coordination. Antimycoplasmal activity of these 1-phenacyl-3-(2-pyridyl)isoquinolines **5a-d** will be compared with the activity of the corresponding amides **3b-e**.

Chemistry

Although the synthesis of 1-amino-3-(6-methyl-2-pyridyl)isoquinoline **2** from 6-methyl-pyridine-2-carbonitrile and 2-methylbenzonitrile has been described by Pijper *et al.* [1] (Scheme I), it appeared to be impossible to obtain comparable yields carrying out the same procedure. Furthermore the work-up procedure appeared to be rather time consuming. So, this compound is synthesized in a slightly different way.

Potassium amide is prepared *in situ* by the addition of potassium to liquid ammonia and subsequently the mixture is cooled to - 78°C. At this temperature 2-methylbenzonitrile in anhydrous diethyl ether and 6-methylpyridine-2-carbonitrile in anhydrous THF are added consecutively. Then the cooling device is removed and the ammonia is allowed to evaporate. After hydrolysis of the residue the product is not isolated by sublimation as described by Pijper but by an extraction procedure. By this method compound **2** was obtained in the same yield as reported by Pijper (34%).

Scheme I.



4a R = CH_3
4b R = C_6H_5

Amides **4a,b** were prepared by acylation of **2** with acyl chlorides (Scheme I) in the same way as described in the previous chapter for the analogous compounds **3a,b** derived from 1-amino-3-(2-pyridyl)isoquinoline. Also in this case yields are rather moderate due to delocalisation of the negative charge of the anion which is formed after treatment of compound **2** with *n*-BuLi. Amides **4a,b** show the same structural features as their analogues **3a,b**. In contrast with N-[3-(6-methyl-2-pyridyl)isoquinolin-1-yl]-acetamide **4a**, N-[3-(6-methyl-2-pyridyl)isoquinolin-1-yl]benzamide **4b** is predominantly present in the iminol form, as can be concluded from both NMR and IR data. Predominance of this iminol form in case of the benzamides **3b** and **4b** is caused by conjugation of the phenyl group with the isoquinoline moiety and is additionally stabilised by formation of an intramolecular hydrogen bond.

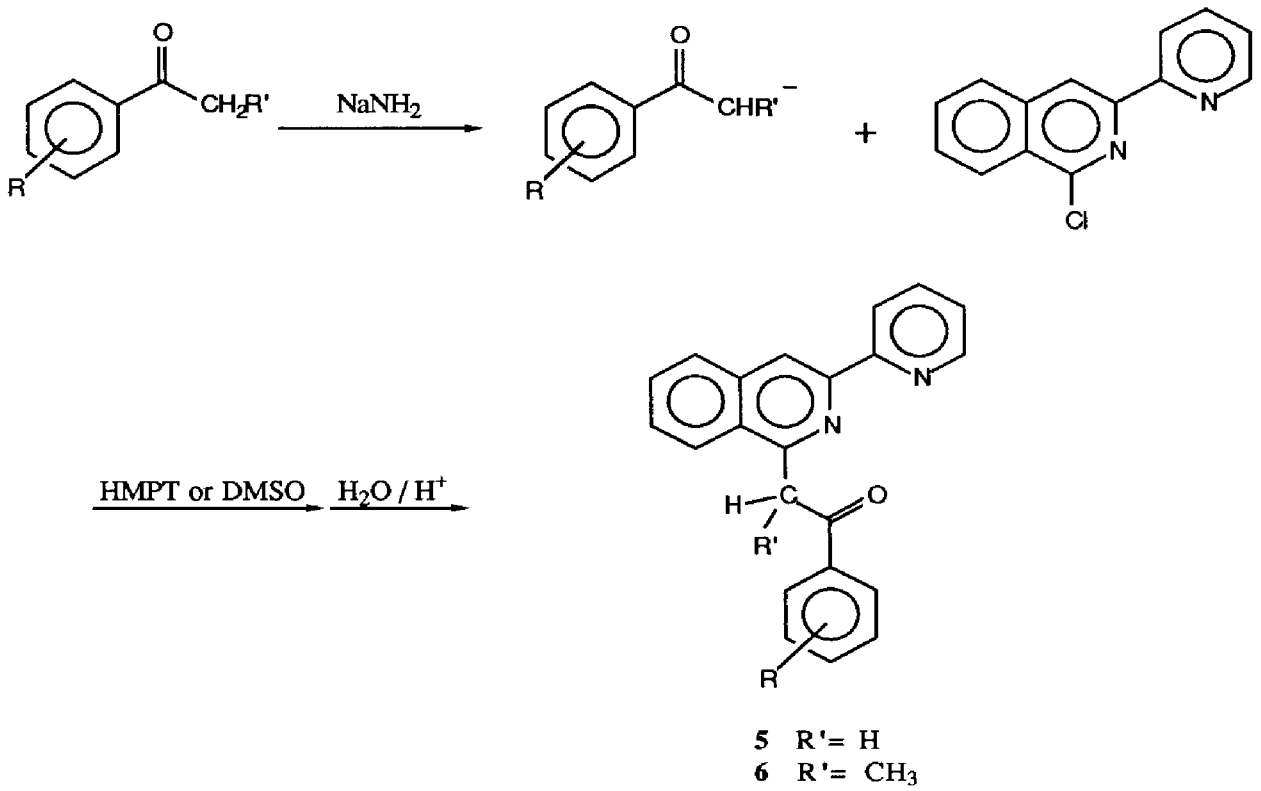
As the chlorine atom of the easy accessible 1-chloro-3-(2-pyridyl)isoquinoline appeared to be sensitive towards nucleophilic substitution, we planned to synthesize 1-phenacyl-3-(2-pyridyl)isoquinolines from this compound and various acetophenones after treating the latter with a base. Although nucleophilic aromatic substitutions may proceed by several different mechanisms only two of them have been described for the reaction of anions of ketones with chlorine substituted quinolines, isoquinolines and quinazolines respectively. Hay and Wolfe [5,6] described the synthesis of 2-phenacylquinolines from 2-chloroquinoline and acetophenones by irradiation of the reaction mixture in the presence of lithium amide in ammonia. They postulated that this reaction proceeds *via* a free radical mechanism. Yields and formation of side products were strongly dependent on the metal amide used. When lithium amide was used 83% of 2-phenacylquinoline was obtained starting from 2-chloroquinoline

whereas this product could not be prepared using potassium amide. Therefore the radical mechanism is highly questionable and moreover application of this method was shown to be restricted to primary ketones.

Synthesis of 4-acylquinazolines from either 4-chloroquinazoline or 4-cyanoquinazoline and various ketones using 50% sodium hydroxide in water has been described by Higashino *et al.* [7,8]. Although 4-acylquinazolines were obtained in this way, hydrolysis of the initially formed product leading to formation of 4-alkylquinazolines was frequently encountered, especially in case of secondary ketones. Using sodium amide in anhydrous benzene didn't solve this problem as the crude reaction mixture had to be poured into ice/water.

According to Coudert *et al.* [9] it is more likely that the formation of 2-(2-oxoalkyl)-quinolines from 2-chloroquinoline proceeds *via* an S_NAr mechanism instead of a radical mechanism and these investigators showed that yields could be improved using a polar aprotic solvent like hexamethylphoshoric triamide (HMPT). The same authors confirmed also the sensitivity of products obtained from secondary ketones towards hydrolysis. From the foregoing it is clear that although various methods have been described for the synthesis of 1-phenacylisoquinolines none of them is satisfactory.

Scheme II.



5 R'= H
6 R'= CH_3

As the procedure described by Coudert *et al.* [9] seemed most promising, synthesis of

1-phenacyl-3-(2-pyridyl)isoquinolines was performed by treatment of the *in situ* prepared anion of acetophenones with 1-chloro-3-(2-pyridyl)isoquinoline in THF/HMPT (Scheme II). In this way we obtained the desired products **5a-d**, however in moderate yields only (22-31%).

Without exception these compounds were isolated as the enol tautomer. This may be concluded from NMR data showing an absorption at 6.76 ppm which can be ascribed to an olefinic hydrogen atom and an absorption at 16.7 ppm which can be assigned to a hydrogen atom participating in an intramolecular hydrogen bond. Also IR data provide evidence for the predominance of this enol tautomer as no carbonyl absorption could be found. The same features have been described for 4-phenacylquinazolines and 2-phenacylquinolines [9]. Besides a keto and enol tautomer the appearance of an enamine tautomer has been described for 2-phenacylquinolines by Yamazaki *et al.* [10] Due to the complexicity of NMR spectra of compounds **5** it was hard to distinguish this tautomer from the enolic form.

Furthermore it was established that only compounds synthesized from primary ketones and 2-chloroquinoline or 4-chloroquinazoline could be obtained as the enol tautomer, whereas analogous products derived from secondary ketones were always present in the keto form. To determine whether the same holds true for compounds obtained from 1-chloro-3-(2-pyridyl)-isoquinoline and secondary ketones, 1-phenyl-2-[3-(2-pyridyl)isoquinolin-1- yl]-1-propanone **6** was synthesized (Scheme II).

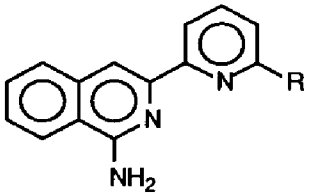
As it appeared very hard to remove HMPT from the reaction mixture, in this synthesis we used DMSO instead. Compound **6** was obtained in low yield and as might be expected from the foregoing the hydrolysis product of this compound viz. 1-ethyl-3-(2-pyridyl)isoquinoline was isolated as well. As the IR spectrum of compound **6** shows a strong absorption at 1690 cm^{-1}, this compound was apparently isolated as the keto tautomer. This finding is in full accordance with what had been found for the analogous 2-quinoline derivative [7]. Even when solved in chloroform compound **6** was present in its keto form, which may be concluded from NMR data showing the absence of an absorption at low field and the presence of characteristic absorptions at 1.8 and 5.54 ppm respectively. Although the enol form of compound **6** apparently is no longer preferred, the occurence of this tautomer is still possible of course. Due to steric hindrance of the methyl group with the isoquinoline nucleus (H-8) it is no longer possible for the enol moiety to be in the same plane as the isoquinoline ring. As a consequence of this, conjugation is no longer appearing and intramolecular hydrogen bond formation does not occur anymore.

Biological activity and structure-activity relationships

All compounds have been tested again with and without the addition of copper to the growth medium. Without the addition of copper, the copper concentration was less than 3 μM. To

determine the copper-dependent antimycoplasmal activity of these compounds, copper was added as $CuSO_4.5H_2O$ to obtain a final concentration of 40 μM.

Table I. MIC Values[a] (μM) against *M. gallisepticum* K514 in a Modified Adler Medium at 37°C.



1,2

Compd	R	without extra copper	extra copper added[b]
Tylosin		0.1	0.1
1	H	452[c]	0.45[c]
2	CH_3	> 25	0.2

[a] Number of determinations of MIC values is two.[b] 40 μM $CuSO_4$.

[c] Values taken from Table I of chapter 3.

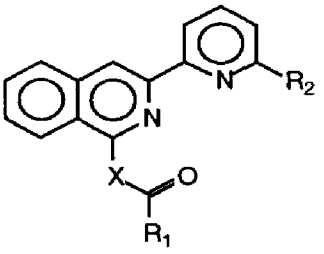
Antimycoplasmal activity was expressed as the minimal inhibitory concentration. MIC values for 1-amino-3-(2-pyridyl)isoquinoline and amides derived thereof are taken from the previous chapter. MIC values for 1-amino-3-(6-methyl-2-pyridyl)isoquinoline **2**, amides **4a** and **4b** derived thereof, 1-phenacyl-3-(2-pyridyl)isoquinolines **5** and 1-phenyl-2-[3-(2-pyridyl)-isoquinolin-1yl]-1-propanone **6** are presented in Tables I and II. When values are preceded by a greater than sign (>) this value represents the highest concentration in which the compound concerned was tested, and which concentration still showed no activity.

From data presented in table I and II it is clear that antimycoplasmal activity of all compounds under investigation is copper dependent. Without addition of extra copper none of them was active against *M.gallisepticum*, whereas in the presence of copper complete growth inhibition was found for all compounds except compound **6** at concentrations varying from 1-10 μM. As we are interested in structure-activity relationships concerning this copper-dependent activity, we will discuss antimycoplasmal activity in the presence of copper only.

Effect of ortho substitution

Although Pijper [1] reports that in the presence of 50 μg/mL Cu^{2+} 1-amino-3-(6-methyl-2-

Table II. MIC Values[a] (μM) against *M. gallisepticum* K514 in a Modified Adler Medium at 37°C.



3-6

Compd	R_1	R_2	X	without extra copper	extra copper added[b]
3a	CH_3	H	NH	> 25[c]	1.9[c]
3b	C_6H_5	H	NH	12[c]	0.1[c]
3c	4-$OCH_3(C_6H_4)$	H	NH	> 160[c]	0.18[c]
3d	4-$Cl(C_6H_4)$	H	NH	> 160[c]	0.35[c]
3e	3,4-$Cl_2(C_6H_3)$	H	NH	> 160[c]	0.64[c]
4a	CH_3	CH_3	NH	> 25	0.78
4b	C_6H_5	CH_3	NH	> 25	0.78
5a	C_6H_5	H	CH_2	> 3	1.5
5b	4-$OCH_3(C_6H_4)$	H	CH_2	> 10	10
5c	4-$Cl(C_6H_4)$	H	CH_2	> 20	5
5d	3,4-$Cl_2(C_6H_3)$	H	CH_2	> 10	5
6	C_6H_5	H	$CH(CH_3)$	> 10	> 10

[a] Number of determinations of MIC values is two.[b] 40 μM $CuSO_4$.

[c] Values taken from Table II and III of chapter 3.

pyridyl)isoquinoline (**2**) was 16 times more active than 1-amino-3-(2-pyridyl)isoquinoline (**1**), we found that compound **2** was only two times more active than compound **1**, as can be seen from table I. This rather large difference might be ascribed to different experimental circumstances like copper content of the growth medium, composition of that medium in general and density of the culture of *M. gallisepticum* at time of inoculation.

Both acylated derivatives of 1-amino-3-(6-methyl-2-pyridyl)isoquinoline (**4a,4b**) are less active than their parent compound **2**, whereas a fourfold increase of antimycoplasmal activity is obtained by benzoylation of 1-amino-3-(2-pyridyl)isoquinoline (**3b**). Pijper assumed that the activity of compound **2** was caused by formation of the lipophilic tetrahedral copper (I)

complex viz. CuL_2^+. Apparently acylation of the amino function of **2** has a detrimental effect on both formation and/or lipophilicity of this complex.

Comparison of antimycoplasmal activity of amides **4a,b** with their analogues **3a,b** shows that introduction of a methyl group at the ortho position of the 3-(2-pyridyl) ring caused an increase of activity in case of the acetamide whereas the opposite is true for the benzamide.

The following consideration might offer an explanation for this finding. In the previous chapter it was shown that antimycoplasmal activity of amides derived from **1** is dependent on the hydrophobic fragmental value of the amide residue and that this dependency is parabolic in nature. Furthermore lipophilicity of the least active amide viz. acetamide **3a** appeared to be too low. So, it is not surprising that enhancement of lipophilicity as a consequence of the introduction of an additional methyl group results in a more active compound viz. acetamide **4a**. As lipophilicity of benzamide **3b** appeared to be almost optimal for antimycoplasmal activity, a further increase of lipophilicity should affect antimycoplasmal activity negatively according to the relationship presented in chapter 3. As can be seen from Table II compound **4b** indeed proved to be less active than its analogue **3b**.

Although there is a considerable difference in lipophilicity of compounds **4a** and **4b**, they appeared to have the same MIC value. This is not surprising at all. Both compounds have a submaximal activity because lipophilicity is either to low in case of **4a** or too high in case of **4b**.

SAR of 1-phenacyl-3-(2-pyridyl)isoquinolines

Antimycoplasmal activity of 1-phenacyl-3-(2-pyridyl)isoquinolines in the presence of copper is rather moderate. The most active compound viz. the unsubstituted α-[3-(2-pyridyl)isoquinolin-1-yl]acetophenone **5a** is 15 times less active than the reference compound tylosin and about as active as the least active amide described in the previous chapter viz. acetamide **3a**.

Within this limited series of compounds **5**, it is not clear how, for example electronic and lipophilic parameters affect the activity. However one thing is clear, any structural change thusfar investigated of the unsubstituted compound **5a** reduces antimycoplasmal activity.

The total lack of activity of compound **6** in the concentration range tested points to the possible importance of the enol tautomer with regard to antimycoplasmal activity.

Furthermore it should be noticed that without exception all 1-phenacyl-3-(2-pyridyl)-isoquinolines **5** are 8-50 times less active than the corresponding amides **3**. So, substitution of the nitrogen atom of the amide moiety by a carbon atom caused a considerable decrease of antimycoplasmal activity. This remarkable difference is most likely due to the presence of the lone-pair electrons at the nitrogen atom of these amides. Considering participation of the oxygen atom in co-ordinating copper, electron density on this atom, which is increased by the

presence of the lone-pair electrons on the nitrogen atom in case of amides, is an important factor.

Conclusions

It can be concluded from this study that acylation of the amino function of 1-amino-3-(6-methyl-2-pyridyl)isoquinoline **2** doesn't result in increased activity against *M.gallisepticum*. Amides **4a,b** derived from 1-amino-3-(6-methyl-2-pyridyl)isoquinoline are less active against *M.gallisepticum* than the analogous amides **3a,b** derived from 1-amino-3-(2-pyridyl)isoquinoline **1**. So, for these compounds di-ortho substitution of the 2,2'-bipyridyl skeleton is not advantageous with regard to antimycoplasmal activity.

Furthermore it was shown that 1-phenacyl-3-(2-pyridyl)isoquinolines could be obtained by nucleophilic substitution of 1-chloro-3-(2-pyridyl)isoquinoline with acetophenone carbanions. Even in the presence of copper 1-phenacyl-3-(2-pyridyl)isoquinolines **5** appeared to be not or only slightly active against *M. gallisepticum*.

As 1-phenacyl-3-(2-pyridyl)isoquinolines are at least 8 times less active than the corresponding N-[3-(2-pyridyl)isoquinolin-1-yl]amides, substitution of NH by CH_2 in the amide moiety of these amides caused a considerable decrease of antimycoplasmal activity. The presence of this amide nitrogen appeared to be essential for antimycoplasmal activity of compounds **3**.

Experimental Section

Chemistry

Melting points were determined with a Mettler FP5/FP52 apparatus. NMR spectra were recorded on a Bruker WH-90 90-MHz spectrophotometer at 21°C. Chemical shifts are expressed in ppm relative to tetramethylsilane. Infrared spectra were recorded on a Perkin-Elmer 580B spectrophotometer. Recording of mass spectra and peak matching were performed with a Varian CH 5DI and a Finnigan MAT 90 mass spectrometer, under electron impact conditions.

All starting materials were commercially available and of the highest purity obtainable. 6-Methylpyridine-2-carbonitrile was obtained by cyanation of 2-picoline-1-oxide with trimethylsilanecarbonitrile and dimethylcarbamoylchloride in dichloromethane [11]. 3,4-Dichloroacetophenone was prepared by Friedel-Crafts acylation of 1,2-dichlorobenzene with aluminum chloride and acetic anhydride [12]. 1-Chloro-3-(2-pyridyl)isoquinoline was prepared from 1-amino-3-(2-pyridyl)isoquinoline (Chapter 3) according to Van der Goot *et al.* [13].

Synthesis

1-Amino-3-(6-methyl-2-pyridyl)isoquinoline (2)

In a thoroughly dried three-necked flask equipped with a mechanical stirrer, 0.1 mol of potassium amide is freshly prepared in 150 mL of ammonia. The mixture is then cooled to -78°C. Subsequently 0.1 mol of 2-methylbenzonitrile in 40 mL of anhydrous diethyl ether is added slowly while the temperature is kept at -78°C. When the addition is complete, a solution of 0.1 mol of 6-methylpyridine-2-carbonitrile in 50 mL of anhydrous THF is added dropwise. Subsequently, the cooling device is removed and stirring is continued to evaporate the ammonia. When the mixture has reached room temperature it was hydrolysed with a small amount of water. After evaporation of the organic phase the pH of the remaining mixture was adjusted to 8 by the addition of some sodium bicarbonate followed by extraction of the water layer with chloroform. The chloroform layer is extracted several times with a 0.15 M sodium citrate buffer pH=1. The water layers are pooled and the pH is adjusted to 8 with sodium hydroxide. The suspension is subsequently extracted with chloroform. After evaporation of the solvent the product is crystallised as the hydrochloride from ethanol:

Yield 9.3 g (34%); mp 262.5-264.0°C;

NMR (DMSO-d6) δ 2.76 (s, 3H, CH_3), 7.56 (d, 1H, $J_{4'-5'}$ = 8.6 Hz, H-5'), 7.82-8.41 (m, 6H, Ar-H), 8.74 (d, 1H, $J_{3'-4'}$ = 8.6 Hz, H-3'), 9.72 (br s, 2H, NH_2).

N-[-3-(6-methyl-2-pyridyl)isoquinolin-1-yl]acetamide (4a)

A suspension of 0.01 mol of 1-amino-3-(6-methyl-2-pyridyl)isoquinoline.HCl in 20 mL of anhydrous THF was stirred under a nitrogen atmosphere and cooled to -10°C. Subsequently 12.5 mL of 1.6 M *n*-butyllithium in hexane was added dropwise and stirring was continued for 10 min. Then 0.02 mol of freshly distilled acyl chloride in 5 mL of anhydrous THF was added, and while the reaction mixture was kept at -10°C, stirring was continued for 1*h*. The ice bath was removed and when the mixture had reached room temperature, it was hydrolysed with water. After evaporation of the organic solvent the pH of the water layer was adjusted to 8 and the water layer was subsequently extracted with chloroform. The chloroform layers were pooled and washed with a dilute sodium bicarbonate solution, dried with anhydrous potassium carbonate, and, after filtration, evaporated to dryness. The residue was washed with diethyl ether and subsequently extracted with boiling chloroform. The extract was evaporated to dryness and the residue was crystallised from ethanol:

Yield 0.5 g (18%) of small white needles, mp 229.5-230.5°C;

NMR ($CDCl_3$) δ 2.59 (s, 3H, CH_3), 2.71 (s, 3H, CH_3), 7.20 (d, 1H, $J_{4'-5'}$ = 7.2 Hz, H-5'), 7.56-8.28 (m, 5H, Ar-H), 8.66 (s, 1H, H-4);

IR (KBr, cm^{-1}) 3220 (NH), 2900 (CH),1660 (C=O),1625, 1570, 1520 (C=C, C=N), 1460, 1435, 1370, 1335, 1260, 960, 890, 800, 755, 740;

MS, m/e 277.1202 (M^+), 277.1215 ($C_{17}H_{15}N_3O$).

N-[3-(6-methyl-2-pyridyl)isoquinolin-1-yl]benzamide (4b)

This compound was synthesized and isolated in the same way as **4a** from 1-amino-3-(6-methyl-2-pyridyl)-isoquinoline and benzoyl chloride. After the washing procedure with diethyl ether the residue was crystallised from methanol/ethyl acetate:

Yield 1.24 g (36%) of light-yellow crystals, mp 183.8-185.3°C;

NMR ($CDCl_3$) δ 2.82 (s, 3H, CH_3), 7.16 (d, 1H, $J_{4'-5'}$ = 7.2 Hz, H-5'), 7.44-7.96 (m, 9H, Ar-H), 8.56 (m, 2H), 9.08 (d, 1H, $J_{3'-4'}$ = 7.2 Hz, H-3'), 16.30 (br s, 1H, OH);

IR (KBr, cm^{-1}) 3600 (OH), 3040 (CH), 1630, 1590, 1570, 1550, 1520 (C=C, C=N), 1465, 1410, 1380, 1335, 1315, 1290, 1140, 1020, 920, 880, 835, 790, 780, 755, 710;

MS, m/e 339.1382 (M^+), 339.1371 ($C_{22}H_{17}N_3O$).

***General procedure for the synthesis of 1-phenacyl-3-(2-pyridyl)isoquinolines* (5)**

In a thoroughly dried three-necked flask equipped with a mechanical stirrer, 20 mL of anhydrous THF is stirred with 3.0 g of a suspension of 50% sodium amide in toluene. While stirring 38 mmol of the respective acetophenone in 20 mL of anhydrous THF is added dropwise. The mixture is kept at 45°C for 2*h*. After the addition of 30 mL hexamethylphosphoric triamide (HMPT), 15 mmol of 1-chloro-3-(2-pyridyl)isoquinoline in 20 mL of anhydrous THF is added dropwise. The mixture is stirred for another hour and subsequently the reaction mixture is poured into ice/water and acidified with concentrated hydrogen chloride. This solution is washed several times with diethyl ether. The remaining water layer is neutralised with sodium bicarbonate to pH = 8, and subsequently extracted with chloroform. The combined chloroform layers were dried with anhydrous potassium carbonate, and, after filtration, evaporated to dryness. Purification is performed through column chromatography using silica gel 60H with diethyl ether/hexane 6:4 (v/v) as eluent. The product is crystallised from 96% ethanol.

α-[3-(2-Pyridyl)isoquinolin-1-yl]acetophenone (5a)

This compound was synthesized from 1-chloro-3-(2-pyridyl)isoquinoline and acetophenone.

Yield 1.5 g (31%); mp 147.5-148.0°C;

NMR ($CDCl_3$) δ 6.76 (s, 1H, CH), 7.26-8.34 (m, 13H, Ar-H), 8.90 (d, $J_{5'-6'}$ = 5.0 Hz, 1H, H-6'), 16.70 (s, 1H, OH);

IR (KBr, cm^{-1}) 3440 (OH), 3050, 3010 (CH), 1595, 1580, 1545, 1535, 1500, 1485, (C=C, C=N), 1475, 1430, 1355, 1250, 1230, 1140, 1065, 1035, 990, 915, 870 (CH o.o.p.), 780, 750, 740, 715, 700, 685;

MS, m/e 324.1250 (M^+), 324.1263 ($C_{22}H_{16}N_2O$).

4-Methoxy-α-[3-(2-pyridyl)isoquinolin-1-yl]acetophenone (5b)

This compound was synthesized from 1-chloro-3-(2-pyridyl)isoquinoline and 4-methoxyacetophenone.

Yield 1.17 g (22%); mp 138.0-138.5°C;

NMR ($CDCl_3$) δ 3.86 (s, 3H, OCH_3), 6.76 (s, 1H, CH), 6.94 and 8.04 (AA'BB' system, J_{ab} = 9.0 Hz, 4H), 7.28-8.28 (m, 8H, Ar-H), 8.84 (d, $J_{5'-6'}$ = 4.5 Hz, 1H, H-6'), 16.60 (s, 1H, OH);
IR (KBr, cm^{-1}) 3450 (OH), 3055, 3000 (CH), 2955, 2935, 2910 (CH_3), 2820 (OCH_3), 1590, 1575, 1545, 1530, 1500, 1480, (C=C, C=N), 1470, 1445, 1350, 1300, 1260, 1240, 1205, 1200, 1165, 1140, 1115, 1100, 1035, 990, 945, 885 (CH o.o.p.), 840, 780, 760, 740, 710, 700, 635, 620, 590, 565, 540;
MS, m/e 354.1333 (M^+), 354.1368 ($C_{23}H_{18}N_2O_2$).

4-Chloro-α-[3-(2-pyridyl)isoquinolin-1-yl]acetophenone (5c)

This compound was synthesized from 1-chloro-3-(2-pyridyl)isoquinoline and 4-chloroacetophenone.
Yield 1.6 g (30%); mp 178.0-179.0°C;
NMR ($CDCl_3$) δ 6.76 (s, 1H, CH), 7.22-8.26 (m, 8H, Ar-H), 7.42 and 8.0 (AA'BB' system, J_{ab}= 9.0 Hz, 4H), 8.84 (d, $J_{5'-6'}$ = 4.5 Hz, 1H, H-6'), 16.70 (s, 1H, OH);
IR (KBr, cm^{-1}) 3440 (OH), 3050, 3010 (CH), 1610, 1590, 1580, 1540, 1530, (C=C, C=N), 1475, 1430, 1355, 1250, 1230, 1140, 1090, 1020, 880 (CH o.o.p.), 840, 780, 745, 730, 705, 525, 470;
MS, m/e 358.0871 (M^+), 358.0873 ($C_{22}H_{15}N_2OCl^{35}$).

3,4-Dichloro-α-[3-(2-pyridyl)isoquinolin-1-yl]acetophenone (5d)

This compound was synthesized from 1-chloro-3-(2-pyridyl)isoquinoline and 3,4-dichloroacetophenone.
Yield 1.8 g (31%); mp 216.5-217.0°C;
NMR ($CDCl_3$) δ 6.74 (s, 1H, CH), 7.26-8.34 (m, 8H, Ar-H), 7.52 (d, J = 9.0 Hz, 1H, Phe H-5), 7.92 (dd, J = 9.0 / 1.8 Hz, 1H, Phe H-6), 8.16 (d, J = 1.8 Hz, 1H, Phe H-2), 8.88 (d, $J_{5'-6'}$ = 4.5 Hz, 1H, H-6'), 16.70 (s, 1H, OH);
IR (KBr, cm^{-1}) 3440 (OH), 3080, 3050, 3010 (CH), 1580, 1560, 1545, 1530, (C=C, C=N), 1470, 1430, 1395, 1350, 1255, 1235, 1145, 1025, 900, 870 (CH o.o.p.), 835, 770, 750, 730,
690, 675, 625;
MS, m/e 392.0471 (M^+), 392.0483 ($C_{22}H_{14}N_2OCl_2{}^{35}$).

1-Phenyl-2-[3-(2-pyridyl)isoquinolin-1yl]-1-propanone (6)

In a thoroughly dried three-necked flask equipped with a mechanical stirrer, 0.05 mol of sodium amide is freshly prepared in 100 mL of ammonia. The ammonia is allowed to evaporate and 30 mL of anhydrous DMSO is added slowly. While keeping the temperature at 45°C, 0.05 mol of propiophenone in 10 mL of anhydrous DMSO is added dropwise. The mixture is stirred for 1.5 *h* at 45°C. Subsequently 0.02 mol of 1-chloro-3-(2-pyridyl)-isoquinoline in 40 mL of anhydrous DMSO is added dropwise and stirring is continued for 30 min. The reaction mixture is poured into ice/water and acidified with concentrated hydrogen chloride. After washing the water layer with diethyl ether the pH is adjusted to 8 by the addition of sodium bicarbonate. Then the mixture is extracted several times with chloroform. The combined chloroform layers are dried with anhydrous potassium carbonate,

and, after filtration, evaporated to dryness. The product is isolated from the remaining oil by preparative TLC using diethyl ether/hexane 6:4 (v/v) as eluent.

Yield 1.28 g (19%); mp 150.0-151.5°C;

NMR ($CDCl_3$) δ 1.80 (d, J = 7.0 Hz, 3H, CH_3), 5.54 (q, J = 7.0 Hz, 1H, CH), 7.14-7.40 (m,4H, Ar-H), 7.62-8.11 (m, 6H, Ar-H), 8.20-8.44 (m, 2H, Ar-H), 8.66 (d, $J_{5'-6'}$ = 5.0 Hz, 1H, H-6'), 8.76 (s, 1H, H-4);

IR (KBr, cm^{-1}) 3090, 3065, 3050 (CH), 2980, 2940 (CH_3), 1690 (C=O), 1620, 1580, 1560, 1495, (C=C, C=N), 1475, 1450, 1425, 1380, 1340, 1330, 1250, 1215, 1000, 950, 825, 800, 785, 760, 745, 715, 685, 645;

MS, m/e 338.1420 (M^+), 338.1419 ($C_{23}H_{18}N_2O$).

Biological activity

Growth inhibition of *M. gallisepticum* K514 was determined in a modified Adler medium at 37°C as described in Chapter 3.

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Chapter 5

SYNTHESIS AND COPPER DEPENDENT ANTIMYCOPLASMAL ACTIVITY OF 1-AMINO-3-(2-PYRIDYL)ISOQUINOLINE DERIVATIVES.2.AMIDINES*

Marcel A.H. de Zwart, Henk van der Goot and Henk Timmerman

Abstract In our search for new compounds with antimycoplasmal activity a series of aromatic amidines derived from 1-amino-3-(2-pyridyl)isoquinoline **1** was synthesized. In the presence of 40 μM copper the most active compounds show growth-inhibition of *M. gallisepticum* in the nanomolar range. These compounds are three times as active as tylosin, an antimycoplasmal therapeutic agent that is used in veterinary practice. In the presence of copper amidines derived from **1** are two to three times more active than the corresponding amides. Furthermore it was established that for these compounds too, the presence of a 2,2'-bipyridyl moiety is a necessary prerequisite for antimycoplasmal activity. As for the amides antimycoplasmal activity of amidines derived from **1** is dependent on the hydrophobic fragmental value of the aromatic nucleus of the amidine moiety. A quantitative structure-activity relationship established the optimal hydrophobic fragmental value of this part of the molecule to be zero.

Introduction

Previous studies from our laboratory revealed that copper complexes of compounds structurally related to 2,2'-bipyridyl are growth inhibitors of *Mycoplasma gallisepticum in vitro* [1-4]. In subsequent studies we established that copper is a very potent inhibitor *in vitro* of NADH-oxidase and lactate dehydrogenase [5], two enzymes involved in the energy providing metabolism of fermentative mycoplasmas [6]. In a proposed mechanism of action of these copper complexes by Smit [7] and Gaisser [8], copper is the ultimate toxic agent, whereas the ligand facilitates the penetration of copper into the cytosol.

After the discovery of the high degree of antimycoplasmal activity of amides and amidines derived from 4-amino-2-(2-pyridyl)quinazoline by Linschoten [4] we focused our attention on the structurally related derivatives of 1-amino-3-(2-pyridyl)isoquinoline. In chapter 3 we have reported on the synthesis and antimycoplasmal activity of both aliphatic and aromatic amides derived from 1-amino-3-(2-pyridyl)isoquinoline. A qualitative structure-activity relationship study initially revealed that antimycoplasmal activity is dependent on the hydrophobic fragmental value of the amide residue. This dependency was parabolic in nature and the optimal hydrophobic fragmental value was established by a subsequent regression analysis to be $\Sigma_f = 0.30$. This value was approximated best by N-[3-(2-pyridyl)isoquinolin-1-yl]benzamide

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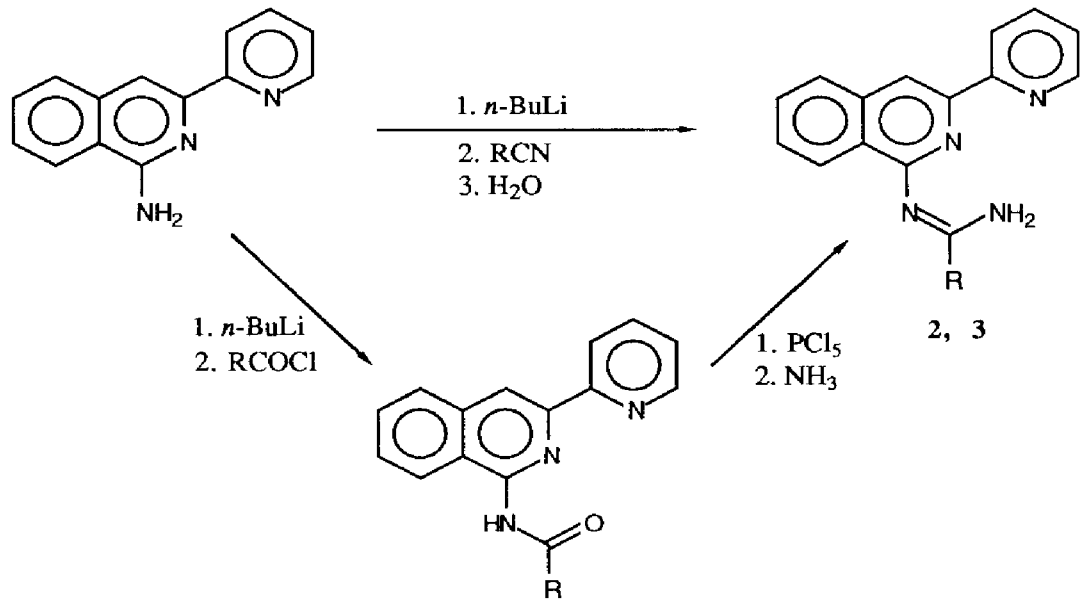
which had a Minimal Inhibitory Concentration (MIC) of 0.1 μM. This value is comparable with the MIC value of tylosin, a macrolide antibiotic which is used in veterinary practice for treatment of mycoplasmal infections in poultry [9].
In this chapter we report on the synthesis and antimycoplasmal activity of amidines derived from 1-amino-3-(2-pyridyl)isoquinoline. Structure optimization was performed according to the Topliss scheme [10]. This was followed by an attempt to establish a quantitative structure-activity relationship.

Chemistry

Basically there are three ways to obtain amidines. They consist of the addition of ammonia or amines to nitriles, to (thio)amides or to imido esters or imido halides [11-14]. Simple addition of ammonia and amines to nitriles are only observed in the case of nitriles activated by electron attracting substituents in the position α to the -CN bond. As an alternative, metal derivatives of ammonia or amines may be used as reactive nucleophiles [15, 16].
When it is not possible to obtain amidines by the addition of amine to nitrile they can be synthesized from the corresponding amide. Treatment of mono N-substituted amides with halogenating agents gives imido halides which react with ammonia to yield the corresponding amidines [17, 18]. As halogenating agent phosphorous pentachloride is preferred.

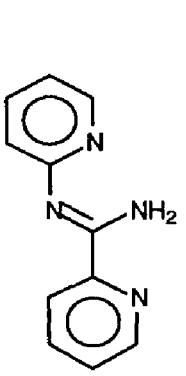
Scheme I.



So, mono N-substituted aromatic amidines **2,3** can be obtained either by direct addition of 1-amino-3-(2-pyridyl)isoquinoline **1** to electron deficient nitriles or treating amides derived from **1** with phosphorous pentachloride and subsequently with ammonia (Scheme I).

In chapter 3 the synthesis of 1-amino-3-(2-pyridyl) isoquinoline **1** and amides derived thereof has been described. 1-Amino-3-(2-pyridyl)isoquinoline **1** was obtained from 2-methyl-benzonitrile and pyridine-2-carbonitrile. Amides could be isolated from the reaction of the anion of 1-amino-3-(2-pyridyl)isoquinoline **1** and various acyl chlorides.

Amidines **2b**, **2c**, **2f** and **2h** were obtained from the corresponding amides. After conversion of these amides with phosphorous pentachloride in anhydrous chloroform to the corresponding imido chlorides, amidines were obtained by the subsequent addition of ammonia. All the other amidines **2a**, **2d**, **2e**, **2g** and **3a-g** were obtained by abstracting a proton from 1-amino-3-(2-pyridyl)isoquinoline **1** with *n*-butyllithium followed by the addition of electron deficient aromatic nitriles to this anion. An attempt to obtain compounds **2b** and **2c** in this way failed. This is in accordance with the electronic influence of the 4-CH_3 and 4-OCH_3 substituent on the carbonitrile moiety. N-(2-pyridyl)-2-pyridinecarboxamidine **4** and N-(5-methyl-3- phenylisoquinolin-1-yl)-2-pyridinecarboxamidine **5** could be obtained by direct addition of 2-pyridinecarbonitrile to the anions of the respective amines.



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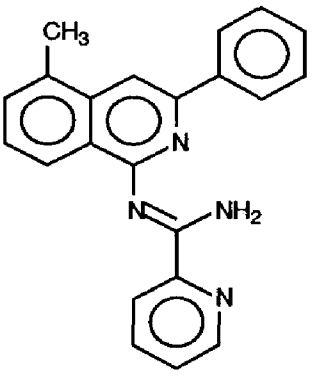

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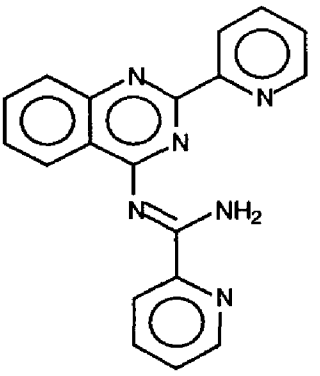


6

Biological activity

Since antimycoplasma activity is apparently copper-dependent for compounds containing a 2,2'-bipyridyl moiety [1, 2], all of the compounds under investigation have been tested with and without the addition of copper to the growth medium. Without the addition of copper, the copper concentration was less than 3 μM [1]. For determination of antimycoplasmal activity of these compounds in the presence of copper, copper was added as $CuSO_4 \cdot 5H_2O$ to obtain a final concentration of 40 μM. The MIC value for copper was established to be 700 μM (Table I). This value is somewhat higher than presented in chapter 3, which is most probably due to minor variations in the growth medium constituents.

MIC values for N-[3-(2-pyridyl)isoquinolin-1-yl)amidines **2a-h**,**3a-g** are presented in tables II and III respectively. All compounds were tested up to a concentration of 100 μM. At

higher concentrations some compounds tend to precipitate when added to growth medium.

Table I. MIC values[a] (μM) against *M.gallisepticum* K514 in a modified Adler medium at 37°C.

Compd	without extra copper	extra copper added[b]
$CuSO_4.5H_2O$	700	-
tylosin	0.1	0.1
1	452	0.45
4	>500	250
5	>100	>100

[a]Number of determinations of MIC values is two. [b]40 μM $CuSO_4$.

In contrast to the structurally related amides, amidines derived from **1** except for compound **2e** posses appreciable antimycoplasmal activity without the addition of extra copper. This activity is most probably due to the small amount of copper, which is present in the growth medium. This idea is supported by the good linear relationship (equation 1) which appeared to exist between the antimycoplasmal activity of compounds **2a-d**, **2f-h** and **3a-g** in the presence of extra copper added (+Cu) and the antimycoplasmal activity of those compounds without any adittional copper present (-Cu).

$$p\,MIC_{(+Cu)} = 0.429\ (\pm 0.070)\ p\,MIC_{(-Cu)} + 4.523\ (\pm 0.438) \qquad (1)$$

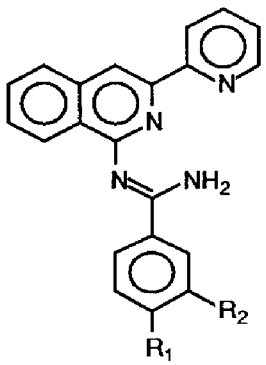
$$n = 14 \qquad r = 0.870 \qquad s = 0.129 \qquad F = 37.446$$

Without the addition of extra copper N-[3-(2-pyridyl)isoquinolin-1-yl)benzamidines are about 100 times more active than the corresponding amides.

In the presence of 40 μM copper all amidines derived from 1-amino-3-(2-pyridyl)-isoquinoline show considerable antimycoplasmal activity. Also in this case antimycoplasmal activity is remarkably enhanced by the addition of a small amount of copper. Thus in the presence of copper, compounds **2a-h** and **3a-g** are about five to thirty times more active than in the absence of copper.

All compounds including **2e** which shows least activity, are more active than the parent compound **1**. In fact the most active compounds **3a** and **3c** are three times more active than the reference compound tylosin, which is used in veterinary practice for treatment of mycoplasmal infections.Benzamidines derived from **1** are two to three times more active than the

Table II. MIC Values[a] (nM)[b] against *M.gallisepticum* K514 in a Modified Adler Medium at 37 °C.



2a-h

Compd	R_1	R_2	without extra copper	extra copper added[c]
2a	H	H	195	63.4
2b	CH_3	H	780	68.3
2c	OCH_3	H	780	104.0
2d	Cl	H	6250	195.0
2e	Cl	Cl	>10000	780.0
2f	H	CH_3	780	48.8
2g	H	OCH_3	1560	78.0
2h	H	Cl	6250	195.0

[a]Number of determinations of MIC values is two. [b]Note that MIC values are given in nanomolar.
[c]40 μM $CuSO_4$.

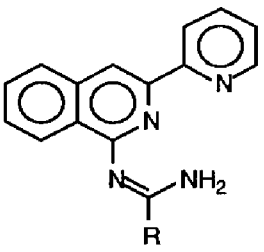
corresponding amides at a copper concentration of 40 μM.

For N-[3-(2-pyridyl)quinazolin-4-yl]-2-pyridinecarboxamidine **6** we found a MIC value 23 times as high as the value reported by Linschoten [4]. This is probably due to the same features, which are also responsible for the increased MIC value for copper. Comparison of the antimycoplasmal activity of the structurally analogous **3a** and **6** shows that at least in this case amidines derived from 1-amino-3-(2-pyridyl)isoquinoline are more active than amidines derived from 4-amino-2-(2-pyridyl)quinazoline.

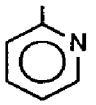
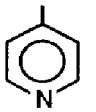
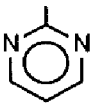
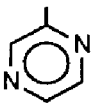
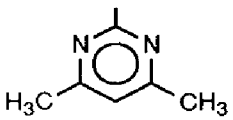
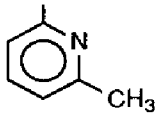
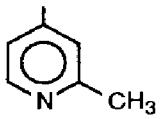
Structure-activity relationships

Just like for a variety of other compounds containing a 2,2'-bipyridyl moiety antimycoplasmal activity of amidines derived from 1-amino-3-(2-pyridyl)isoquinoline is copper dependent.Due to this remarkable copper effect it is very likely that these compounds also act via their copper

Table III. MIC Values[a] (nM) against *M.gallisepticum* K514 in a Modified Adler Medium at 37°C.



3a-g

Compd	R	without extra copper	extra copper added[c]
3a		390	32.5
3b		390	39.0
3c		195	32.5
3d		195	39.0
3e		195	45.5
3f		390	65.0
3g		390	65.0

[a]Number of determinations of MIC values is two.

[b]Note that MIC values are given in nanomolar.

[c]40μM $CuSO_4$.

complexes.

As proposed for the corresponding amides the increase in activity of these amidines compared to the parent compound **1** may be due to the presence of a third coordination site for

the copper atom. In fact it is known that amidines are able to participate as a ligand in copper complexes [19].

To verify the correctness of this hypothesis we investigated antimycoplasmal activity of N-(2-pyridyl)-2-pyridinecarboxamidine **4** and N-[5-methyl-3-phenylisoquinolin-1-yl]-2-pyridinecarboxamidine **5**, two amidines comparable with the ones under investigation but lacking a 2,2'-bipyridyl moiety. Surprisingly these compounds have negligible antimycoplasmal activity (Table I). So, although we cannot exclude participation of the amidine moiety in complex formation, contribution of this part of the molecule is small compared to that of the 2,2'-bipyridyl moiety.

Amidines exist as a mixture of the imino and the amino tautomer with N-aryl substituted derivatives occuring predominantly in the amino form [20-22]. Since we are only dealing with N-aryl substituted amidines we may consider this part of the molecule i.e. the 3-(2-pyridyl)-isoquinoline part with the amidine moiety attached to it to be constant for all compounds when structure-activity relationships are considered. So, for both quantitative and qualitative considerations of a possible structure-activity relationship we only take into account the influence of the part of the molecule which is varied within these series viz. the aromatic nucleus of the amidine moiety symbolized by R in the general form **2,3** (Scheme I).

Since antimycoplasmal activity of these compounds is copper dependent for structure-activity relationship considerations we focus our attention to antimycoplasmal activity in the presence of copper.

When we consider the activity sequence of the original Topliss series, that is compounds **2a-e**, an increase of activity is paralleled by a decrease in lipophilicity of the aromatic nucleus. When we compare antimycoplasmal activity of the 4-substituted benzamidines **2b-d**, with the corresponding 3-substituted benzamidines **2f-h** it is obvious that the position of the substituent in the aromatic nucleus has no influence on biological activity. As a consequence of this dependency on lipophilicity a further increase of antimycoplasmal activity could be achieved by making the compounds less lipophilic. Therefore we decided to synthesize some N-heterocyclic aromatic amidines (**3a-g**). Indeed, by doing so we did establish an increase of antimycoplasmal activity. As for the benzamidines a decrease of lipophilicity is paralleled by an increase of antimycoplasmal activity of these N-heterocyclic aromatic amidines. However when lipophilicity is decreased further, antimycoplasmal activity does not increase anymore. The very hydrophilic compounds **3c** and **3d** show antimycoplasmal activity comparable to the far more lipophilic analogues **3a** and **3b**. So, this qualitative approach to a structure-activity relationship suggests the existence of an optimal lipophilicity for antimycoplasmal activity of these amidines. In chapter 3, an analogous dependency was found for antimycoplasmal activity of amides derived from 1-amino-3-(2-pyridyl)isoquinoline **1**.

In an attempt to establish a quantitative structure-activity relationship we tried to find a correlation between the antimycoplasmal activity and lipophilicity. Parameters chosen were MIC values for biological activity and hydrophobic fragmental values (Σ_f) for lipophilicity. Hydrophobic fragmental values were calculated for the substituted aromatic nucleus according to Rekker (Table IV) [23].

Table IV. Hydrophobic Fragmental Values.[a]

Compd	R	Σ_f	MIC_{calcd}(nM)[b]	MIC_{obsd}(nM)
2a	C_6H_5	1.840	88.5	63.4
2b	4-$CH_3(C_6H_4)$	2.359	156.8	68.3
2c	4-$OCH_3(C_6H_4)$	1.920	95.8	104.0
2d	4-$Cl(C_6H_4)$	2.582	209.4	195.0
2e	3,4-$(Cl)_2(C_6H_3)$	3.324	662.0	780.0
2f	3-$CH_3(C_6H_4)$	2.359	156.8	48.8
2g	3-$OCH_3(C_6H_4)$	1.920	95.8	78.0
2h	3-$Cl(C_6H_4)$	2.582	209.4	195.0
3a	C_5H_4N	0.520	39.1	32.5
3b	C_5H_4N	0.520	39.1	39.0
3c	$C_4H_3N_2$	-0.380[c]	37.8	32.5
3d	$C_4H_3N_2$	-0.380[c]	37.8	39.0
3e	3,5-$(CH_3)_2C_4HN_2$	0.658	40.8	45.5
3f	CH_3-C_5H_3N	1.040	48.3	65.0
3g	CH_3-C_5H_3N	1.040	48.3	65.0

[a]See ref 23. [b]Calculated from equation 3. [c]Personal communication R. F. Rekker.

By multiple regression analysis the following equation is obtained:

$$-\log MIC = 7.454\ (\pm 0.065) - 0.101\ (\pm 0.014)\ (\Sigma_f)^2 \qquad (2)$$

$$n = 15 \qquad r = 0.892 \qquad s = 0.174 \qquad F = 50.566$$

When compounds **2b** and **2f** are omitted for legitimate statistical reasons (residual > 2x standard deviation) a much better equation is obtained:

$$-\log MIC = 7.439\ (\pm 0.033) - 0.114\ (\pm 0.007)\ (\Sigma_f)^2 \qquad (3)$$

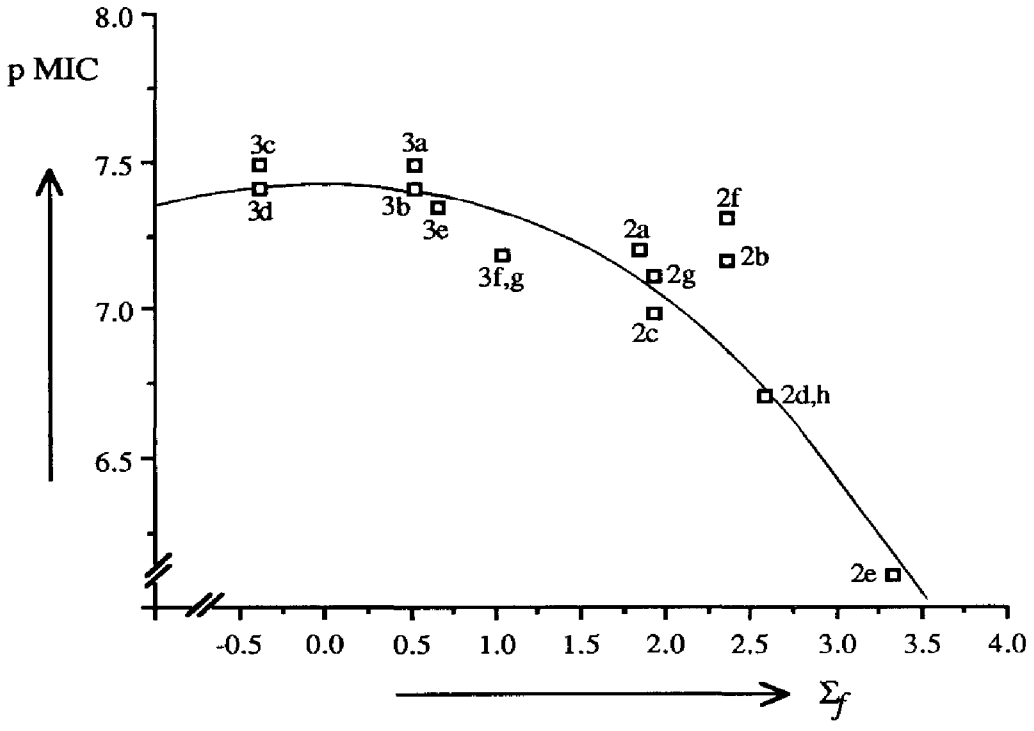
n = 13 r = 0.978 s = 0.087 F = 236.369

So for thirteen of the original series of fifteen compounds a very good correlation is found between antimycoplasmal activity and hydrophobic fragmental values of the aromatic nucleus of the amidine moiety.

As we did not observe anomalous physico-chemical properties for both methyl-substituted compounds **2b** and **2f** compared to other benzamidines synthesized, we have no explanation for the fact that these compounds have to be omitted for statistical reasons from the equation obtained. It is not very likely that this anomalous behaviour can be ascribed to electronic features, since we didn't find any correlation between antimycoplasmal activity and an electronic parameter like σ.

The dependency of antimycoplasmal activity on hydrophobic fragmental values is consistent with a parabolic nature, which means that an optimal lipophilicity for antimycoplasmal activity exists (Figure I).

Figure I. p MIC vs Σ_f. For the identity of **2a-3g**, see Table II and III.



However, as data on the left side of the parabola are not included in regression analysis, another relationship is also possible, e.g. of such a nature that in this range of Σ_f values (i.e. Σ_f < -0.38) antimycoplasmal activity does not decrease with increasing hydrophilicity. According to the equation obtained the optimal contribution to lipophilicity of the substituted aromatic nucleus is $\Sigma_f = 0$. Realizing that the optimum for lipophilicity is rather broad and taking into account the difference in lipophilicity between aromatic amides and corresponding amidines (Σ_f amide - Σ_f amidine = 0.5) this value corresponds very well with the optimal lipophilicity that was found for antimycoplasmal activity of amides derived from **1**.
So, apparently this optimal lipophilicity with regard to antimycoplasmal activity is more generally applicable to compounds derived from **1**.

Conclusions

For amidines derived from **1**, the presence of a 2,2'-bipyridyl moiety is a necessary prerequisite for antimycoplasmal activity.
In the presence of a small non-toxic amount of copper amidines derived from **1** are very potent antimycoplasmal agents with MIC values in the nanomolar range. Even without the addition of extra copper some of these compounds show complete growth-inhibition in the micromolar range.

In the presence of copper amidines are two to three times as active as the corresponding amides.

Antimycoplasmal activity of amidines derived from **1** is dependent on lipophilicity as was found for the structurally related amides and this dependency is parabolic in nature. A quantitative structure-activity relationship was established, which revealed that the optimal hydrophobic fragmental value of the substituted aromatic nucleus of the amidine moiety is zero.

Experimental Section

Chemistry

Melting points were determined using a Mettler FP5/FP52 apparatus.

NMR spectra were recorded on a Bruker WH-90 90 MHz spectrophotometer at 21°C. Chemical shifts are expressed in ppm relative to tetramethylsilane. Infrared spectra were recorded on a Jasco IRA II spectrophotometer. Recording and peak matching of mass spectra were performed with a Varian CH 5 DI mass spectrometer, electron impact 70 eV. All starting materials were commercially available and of the highest purity obtainable. 1-Amino-3-(2-pyridyl)isoquinoline and amides derived thereof were synthesized as described in chapter 3.

1-Amino-5-methyl-3-phenylisoquinoline was taken from the laboratory stock [24]. 3,4-Dichlorobenzonitrile was synthesized from the corresponding aldehyde [25]. Pyrimidine-2-carbonitrile and 3,5-dimethylpyrimidine-2-carbonitrile were synthesized from the corresponding 2-chloro compounds by treatment of the quarternary ammonium salt, prepared from these compounds and trimethylamine, with potassium cyanide in acetamide [26, 27]. 6-Methylpyridine-2-carbonitrile and 6-methylpyridine-4-carbonitrile were obtained by cyanation of 2-picoline-1-oxide [28, 29]. Analytical results for compounds indicated by the molecular formula were within ± 0.4% of the theoretical values.

Synthesis

General procedure for the synthesis of amidines from 1-amino-3- (2-pyridyl)isoquinoline and electron deficient nitriles (2a,2d,2e,2g)

A solution of 0.05 mol 1-amino-3-(2-pyridyl)isoquinoline **1** in 100 mL anhydrous THF was stirred under a nitrogen atmosphere and cooled to -10°C. Subsequently 31.25 mL 1.6 M *n*-butyllithium in hexane was added dropwise and stirring was continued for ten minutes.

Then 0.05 mol nitrile in a minimal amount of THF was added and, while keeping the reaction mixture at -10°C, stirring was continued for ten minutes. When the mixture had reached room temperature, it was reluxed for several hours varying from 2-10 *h* dependent on the nitrile. After cooling the mixture was hydrolyzed by the addition of a small amount of water. The organic phase was evaporated and the remaining water layer was extracted with chloroform, after adjusting the pH to 8 with a dilute bicarbonate solution. The combined chloroform layers were dried with anhydrous potassium bicarbonate and, after filtration, evaporated to dryness.

General procedure for the synthesis of amidines from corresponding amides derived from 1-amino-3-(2-pyridyl)isoquinoline (2b,2c,2f,2h)

A solution of 0.02 mol phosphorous pentachloride in 50 mL freshly distilled chloroform was stirred under a

nitrogen atmosphere at room temperature. A solution of 0.01 mol amide in 50 mL chloroform was added dropwise and the mixture was refluxed for half an hour. The mixture was cooled in an ice bath and anhydrous ammonia was bubbled through the mixture for one hour. Subsequently an ice cold saturated bicarbonate solution was added slowly. The chloroform layer was separated, washed three times, dried with anhydrous potassium carbonate and, after filtration, evaporated to dryness.

N-[3-(2-pyridyl)isoquinolin-1-yl]benzamidine (2a)

This compound was synthesized from 1- amino-3-(2-pyridyl)isoquinoline and benzonitrile. Time of reflux was 4*h*. The crude reaction mixture was washed with a little methanol to remove traces of unreacted starting material. The remaining solid material was filtered off and crystallized from $CHCl_3$/ p.e. 60-80: yield 4.1 g (25%); mp 184.7-186.7°C;

NMR ($CDCl_3$) δ 7.36 (m, 1H, 2-pyr H-5), 7.54-8.0 (m, 7.5 H), 8.13-8.30 (m, 3H),8.42 (s, 1H, H-4), 8.79 (d, J = 4.5 Hz,1H, 2-pyr H-6), 9.06 (m,1H, 2-pyr H-3);

IR (KBr,cm^{-1}) 3400(NH), 3040(CH), 1605, 1570, 1560, 1540, 1520(C=C,C=N), 1470, 1460, 1440, 1420, 1380, 1330, 1140, 1045, 1025, 925, 900, 880, 780, 750, 740, 700, 690, 620, 580;

MS, m/e 324.1368 (M^+), 324.1375 ($C_{21}H_{16}N_4$). Anal.($C_{21}H_{16}N_4$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl)-4-methylbenzamidine (2b)

This compound was synthesized from the corresponding amide. The crude product was crystallized from $CH_3OH/CH_3COOC_2H_5$: yield 1.9 g (60%); mp 188.1-188.3 °C;

NMR ($CDCl_3$) δ 2.44 (s, 3H, CH_3), 7.29 (m, 1H, 2-pyr H-5), 7.33 and 8.06 (AA'BB' system, J_{ab} = 8.0 Hz, 4H, Phe H), 7.54-7.95 (m, 5H), 8.21 (d, J = 8.0 Hz, 1H, H-8), 8.39 (s, 1H, H-4), 8.76 (d, J = 4.5 Hz,1H, 2-pyr H-6), 9.03 (m, 1H, 2-pyr H-3);

IR (KBr,cm^{-1}) 3450(NH), 3060(CH), 1600(s), 1575, 1560, 1545, 1520, 1495(C=C,C=N), 1475, 1420, 1380, 1330, 1245, 1180, 1145, 1045, 1015, 990, 965, 920, 900, 880, 860, 840, 825, 785, 755, 740, 690, 670, 640, 615, 580;

MS, m/e 338.1522 (M^+), 338.1531 ($C_{22}H_{18}N_4$). Anal. ($C_{22}H_{18}N_4$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl]-4-methoxybenzamidine (2c)

This compound was synthesized from the corresponding amide. The crude reaction mixture was purified via column chromatography using silica gel 60 H with $CHCl_3/CH_3COOC_2H_5/NH_3$ (10:10:1) as eluent. The fractions containing **2c** were pooled and after evaporation of the solvent, the product was crystallized from $CH_3OH/CH_3COOC_2H_5$: yield 0.85 g (24%); mp 199.3-199.6 °C;

NMR ($CDCl_3$) δ 3.90 (s, 3H, OCH_3), 7.05 and 8.12 (AA'BB' system, J_{ab} = 10.0 Hz, 4H, Phe H), 7.30 (m, 1H, 2-pyr H-5), 7.50-7.96 (m, 5H), 8.21 (d, J = 8.0 Hz, 1H, H-8), 8.38 (s, 1H, H-4), 8.76 (d, J = 4.5Hz, 1H, 2-pyr H-6), 9.0 (m, 1H, 2-pyr H-3);

IR (KBr,cm^{-1}) 3330(NH), 3160, 3040(CH), 3000, 1610, 1575, 1530, 1500(C=C,C=N), 1480, 1470, 1415, 1380, 1365, 1330, 1305, 1245, 1180,
1165, 1140, 1025, 995, 955, 925, 885, 860, 835, 785, 755, 740, 690, 675, 640, 580, 560, 545, 525;
MS, m/e 354.1480 (M^+), 354.1480 ($C_{22}H_{18}N_4O$). Anal. ($C_{22}H_{18}N_4O$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl]-4-chlorobenzamidine (2d)

This compound was synthesized from 1-amino-3-(2-pyridyl)isoquinoline and 4-chlorobenzonitrile. Time of reflux was 6*h*. The precipitate that appears after hydrolysis was filtered off and crystallized from $CHCl_3$/ p.e.60-80: yield 9.2 g (51%); mp 162.0-163.0 °C;
NMR ($CDCl_3$) δ 7.33 (m, 1H, 2-pyr H-5), 7.53 and 8.11 (AA'BB' system, J_{ab} = 8.0 Hz, 4H, Phe-H), 7.50-8.08 (m, 5H), 8.22 (d, J = 8.0 Hz, 1H, H-8), 8.42 (s, 1H, H-4), 8.78 (m, J = 4.8 Hz, 1H, 2-pyr H-6), 9.0 (m,1H, 2-pyr H-3);
IR(KBr,cm^{-1}) 3480(NH), 3050 (CH), 1610, 1580, 1560, 1550, 1540, 1510(C=C,C=N), 1485, 1470, 1445, 1425, 1395, 1380, 1365, 1335, 1140, 1090, 1050, 1010, 990, 960, 940, 885, 860, 845, 790, 775, 750, 680, 620, 580;
MS, m/e 358.0967 (M^+), 358.0985 ($C_{21}H_{15}ClN_4$,^{35}Cl). Anal. ($C_{21}H_{15}ClN_4$) C, H, N, Cl.

N-[3-(2-pyridyl)isoquinolin-1-yl]-3,4-dichlorobenzamidine (2e)

This compound was synthesized from 1-amino-3-(2-pyridyl)isoquinoline and 3,4-dichlorobenzonitrile and isolated in the same way as compound **2d**.Time of reflux was 2*h*. Yield 6.5 g (33%); mp 180.0-181.0 °C;
NMR ($CDCl_3$) δ 7.30 (m, 1H, 2-pyr H-5), 7.50-7.95 (m, 5H), 7.55 (d, J = 8.0 Hz,1H, Phe H-5),7.88 (d, J = 8.0 Hz, 1H, Phe H-6), 8.15 (d, J = 8.0 Hz, 1H, H-8),8.23 (d, J = 1.8 Hz,1H, Phe H-2),8.40 (s, 1H, H-4), 8.76 (d, J = 4.8 Hz, 1H, 2-pyr H-6), 8.93 (m, 1H,2-pyr H-3);
IR (KBr,cm^{-1}) 3460(NH), 3050(CH), 1610, 1580, 1560, 1545, 1505(C=C,C=N), 1480, 1465, 1420, 1385, 1365, 1345, 1240, 1200, 1170, 1145, 1020, 990, 960, 930, 905, 885, 865, 830, 790, 755, 740, 720, 665, 620, 580;
MS, m/e 392.0582 (M^+), 392.0595 ($C_{21}H_{14}Cl_2N_4$, ^{35}Cl). Anal. ($C_{21}H_{14}Cl_2N_4$) C, H, N; Cl : calcd, 18.03; found, 18.48.

N-[3-(2-pyridyl)isoquinolin-1-yl)-3-methylbenzamidine (2f)

This compound was synthesized from the corresponding amide. The crude reaction mixture was purified via column chromatography using silica gel 60 H with $CHCl_3/CH_3COOC_2H_5/NH_3$ (10:10:1) as eluent. The fractions containing **2f** were pooled and after evaporation of the solvent, the product was crystallized from $CH_3OH/CH_3COOC_2H_5$: yield 1.05 g (31%); mp 153.3-155.0 °C;
NMR ($CDCl_3$) δ 2.50 (s, 3H, CH_3), 7.31 (m, 1H, 2-pyr H-5), 7.39-8.0 (m,10H), 8.24 (d, J =8.0 Hz, 1H, H-8), 8.42 (s, 1H, H-4), 8.78 (d, J = 4.8 Hz, 1H, 2-pyr H-6), 9.05 (m,1H, 2-pyr H-3);

IR (KBr,cm^{-1}) 3400 (NH), 3040(CH), 1600, 1580, 1560, 1525(C=C,C=N), 1485, 1470, 1425, 1380, 1330, 1145, 1080, 1045, 990, 960, 925, 885, 860, 790, 745, 700, 675, 620, 585, 525;
MS, m/e 338.1539 (M^+), 338.1531 ($C_{22}H_{18}N_4$). Anal. ($C_{22}H_{18}N_4$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl)-3-methoxybenzamidine (2g)

This compound was synthesized from 1-amino-3-(2-pyridyl)isoquinoline and 3-methoxybenzonitrile. Time of reflux was 10*h*. A little diethyl ether was poured on the crude reaction mixture to obtain yellow solid material from the black oil. The precipitate was isolated by filtration and crystallized from $CH_3OH/CH_3COOC_2H_5$: yield 5.5 g (31%); mp 149.4-149.6 ^oC;
NMR ($CDCl_3$) δ 3.94 (s, 3H, OCH_3), 7.11(ddd, J = 7.8, 2.5, 1.8 Hz, 1H, Phe H-6), 7.26-7.40 (m, 1H, 2-pyr H-5), 7.45-7.96 (m, 8H), 8.22 (d, J = 8.0 Hz, 1H, H-8), 8.42 (s, 1H, H-4), 8.77 (d, J = 4.8 Hz, 1H, 2-pyr H-6), 9.02 (m, 1H, 2-pyr H-3);
IR (KBr,cm^{-1}) 3400(NH), 3040(CH), 1600, 1575, 1510(C=C,C=N), 1480, 1470, 1420, 1380, 1365, 1342, 1310, 1285, 1235, 1190, 1160, 1140, 1080, 1045, 990, 960, 925, 880, 855, 810, 780, 747, 738, 700, 672, 618, 565, 500, 470;
MS, m/e 354.1452 (M^+), 354.1480 ($C_{22}H_{18}N_4O$). Anal. ($C_{22}H_{18}N_4O$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl)-3-chlorobenzamidine (2h)

This compound was synthesized from the corresponding amide. The crude product was crystallized twice from $CH_3OH/CH_3COOC_2H_5$: yield 2.9 g (80%); mp 147.6-148.2 ^oC;
NMR ($CDCl_3$) δ 7.31 (m, 1H, 2-pyr H-5), 7.44-8.17 (m, 8H), 8.17 (s, 1H, Phe H-2), 8.21 (d, J = 8.0 Hz, 1H, H-8), 8.42 (s, 1H, H-4), 8.78 (d, J = 4.8 Hz, 1H, 2-pyr H-6), 8.99 (m, 1H,2-pyr H-3);
IR (KBr,cm^{-1}) 3400(NH), 3040(CH), 1600, 1578, 1555, 1535, 1510(C=C,C=N), 1480, 1470, 1420, 1405, 1380, 1330, 1140, 1090, 1075, 1045, 1025, 1010, 990, 925, 900, 885, 860, 785, 755, 748, 720, 670, 618, 582, 520, 445;
MS, m/e 358.0980 (M^+), 358.0985 ($C_{22}H_{15}ClN_4$, ^{35}Cl). Anal. ($C_{22}H_{15}ClN_4$) C, H, N, Cl.

N-[3-(2-pyridyl)isoquinolin-1-yl)-2-pyridinecarboxamidine (3a)

This compound was synthesized from 1-amino-3-(2-pyridylisoquinoline and 2-pyridine- carbonitrile. Time of reflux was 2*h*. The crude reaction mixture was crystallized from $CHCl_3$/ p.e.60-80: yield 8.1 g (50%); mp 185.0-186.0 ^oC;
NMR ($CDCl_3$) δ 7.22-8.00 (m, 7H), 8.20-8.44 (br, 1H, NH), 8.23 (d, J = 8.0 Hz,1H, H-8), 8.40 (s, 1H, H-4), 8.61-8.86 (m, 3H), 9.01 (m, 1H, 2-pyr H-3), 10.95 (br, 1H, NH);
IR (KBr,cm^{-1}) 3380(NH), 3050(CH), 1615, 1580, 1560, 1545(C=C,C=N), 1480, 1465, 1435, 1385, 1365, 1330, 1245, 1200, 1140, 1040, 990, 960, 925, 895, 885, 860, 800, 785, 755, 740, 705, 675, 650, 620, 560, 550, 525;

MS, m/e 325.1322 (M^+), 325.1327 ($C_{20}H_{15}N_5$). Anal. ($C_{20}H_{15}N_5$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl)-4-pyridinecarboxamidine (3b)

This compound was synthesized and isolated in the same way as compound **3a**: yield 13.8 g (85%); mp 221.5-222.3 °C;

NMR ($CDCl_3$) δ 7.36 (m, 1H, 2-pyr H-5), 7.57-8.05(m, 5H), 8.0 and 8.85 (AA'BB' system, J_{ab} = 6.1 Hz, 4H, 4-pyr H), 8.22(d, J = 8.0 Hz, 1H, H-8), 8.44 (s, 1H, H-4), 8.73-8.87 (m, 1H, 2-pyr H-6), 8.98 (m, 1H, 2-pyr H-3);

IR (KBr,cm^{-1}) 3300(NH), 3050(CH), 1630, 1580, 1550, 1530, 1520(C=C,C=N), 1480, 1470, 1425, 1410, 1385, 1365, 1345, 1145, 1060, 1030, 995, 955, 935, 840, 785, 750, 745, 730, 705, 670, 620, 585, 560, 525;

MS, m/e 325.1336 (M^+), 325.1327($C_{20}H_{15}N_4$). Anal.($C_{20}H_{15}N_4$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl)-2-pyrimidinecarboxamidine (3c)

This compound was synthesized from 1-amino-3-(2-pyridyl)isoquinoline and 2-pyrimidinecarbonitrile. Time of reflux was 8*h*. The crude reaction mixture was acidified with concentrated hydrochloric acid and extracted with diethyl ether. Then the water layer was neutralised with sodium bicarbonate to pH 8.0 and extracted with diethyl ether. This organic layer contained mainly the major side product formed during this reaction. Subsequently, the water layer was extracted with chloroform. This chloroform layer was dried with anhydrous sodium carbonate, filtered and evaporated to dryness. The residue was crystallized from ethanol/diethyl ether.The precipitate formed was filtered off and the filtrate was evaporated. The residue was crystallized from $CHCl_3$/ p.e.60-80. The precipitate consisted for the most part of the starting compound 1-amino-3-(2-pyridyl)- isoquinoline. Again the mother liquor was evaporated and a small volume of ethanol was poured on the residue. The solid material formed was filtered off and crystallized from ethanol: yield 2.1 g (13%); mp 182.3-183.8 °C;

NMR ($CDCl_3$) δ 7.31 (m, 1H, 2-pyr H-5), 7.46 (t, J = 5.0 Hz, 1H, pyrimidyl H-4), 7.61-7.97 (m, 6H), 8.07-8.36 (br s, 1H, NH), 8.26 (d, J = 8.0 Hz, 1H, H-8), 8.47 (s, 1H, H-4), 8.78 (d, J = 4.5 Hz, 1H, 2-pyr H-6), 8.99 (d, J = 5.0 Hz, 2H, pyrimidyl H-3 and H-5), 9.08 (m, 1H, 2-pyr H-3), 11.08 (br s, 1H, NH);

IR (KBr,cm^{-1}) 3440(NH), 3040(CH), 1615, 1575, 1555, 1540(C=C,C=N), 1480, 1420, 1395, 1380, 1360, 1330, 1265, 1220, 1180, 1140, 1040, 1020, 1010, 990, 965, 925, 885, 860, 820, 785, 760, 745, 705, 670, 630, 620, 590, 570, 560, 525, 470;

MS, m/e 326.1284 (M^+), 326.1280 ($C_{19}H_{14}N_6$). Anal. ($C_{19}H_{14}N_6$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl)-2-pyrazinecarboxamidine (3d)

This compound was synthesized from 1-amino-3-(2-pyridyl)isoquinoline and 2-pyrazine- carbonitrile. Time of reflux was 4*h*. The crude product was crystallized from $CH_3OH/CH_3COOC_2H_5$: yield 15.5 g (95%); mp 231.5-232.0 °C;

NMR ($CDCl_3$) δ 7.32 (m, 1H, 2-pyr H-5), 7.62-7.97 (m, 5H), 8.20 (d, J = 8.0 Hz,1H, H-8), 8.44 (s, 1H, H-4),

8.61 (dd, J = 2.7, 1.4 Hz, 1H, 2-pyrazinyl H-5), 8.77 (m, 2H, 2-pyr H-6 and 2-pyrazinyl H-6), 9.06 (m, 1H, 2-pyr H-3), 10.05 (d, 1H, J = 1.4 Hz, 2-pyrazinyl H-3), 11.08 (br s, 1H, NH);
IR (KBr,cm^{-1}) 3400(NH), 3050(CH), 1625, 1575, 1560, 1550, 1540(C=C,C=N), 1485, 1465, 1425, 1380, 1360, 1345, 1170, 1145, 1015, 925, 890, 850, 785, 765, 750, 730, 715, 625, 615, 525, 495;
MS, m/e 326.1284 (M^+), 326.1280 ($C_{19}H_{14}N_6$). Anal. ($C_{19}H_{14}N_6$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl)-3,5-dimethyl-2-pyrimidinecarboxamidine (3e)
This compound was synthesized from 1-amino-3-(2-pyridyl)isoquinoline and 3,5-dimethyl-2-pyrimidine-carbonitrile. Time of reflux was 4*h*. After hydrolysis of the reaction mixture, the organic phase was evaporated and some diethyl ether was poured on the remaining water layer, resulting in precipitation of the crude product. The precipitate was filtered off and crystallized from ethanol. The pure compound was obtained after recrystallization from ethanol: yield 6.8 g (39%); mp 195.5-197.0 ^oC;
NMR ($CDCl_3$) δ 2.66 (s, 6H, CH_3), 7.16 (s, 1H, pyrimidyl H-4), 7.30 (m, 1H, 2-pyr H-5), 7.56-7.96 (m, 4H), 8.26 (d, J = 8.0 Hz, 1H, H-8), 8.46 (s, 1H), 8.76 (d, J = 4.5 Hz, 1H, 2-pyr H-6), 9.06 (m, 1H, 2-pyr H-3),11.19 (br s, 1H, NH), 8.11-8.47 (br s, 1H, NH);
IR (KBr,cm^{-1}) 3420, 3040(CH), 1620, 1580, 1560, 1540(C=C,C=N), 1480, 1420, 1380, 1360, 1345, 1330, 1280, 1140, 1030, 990, 960, 925, 905, 865, 805, 785, 765, 740, 730, 720,670, 620, 580, 570, 555, 535, 485;
MS, m/e 354.1571 (M^+), 354.1593 ($C_{21}H_{18}N_6$). Anal.($C_{21}H_{18}N_6$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl)-6-methyl-2-pyridinecarboxamidine (3f)
This compound was synthesized from 1-amino-3-(2-pyridyl)isoquinoline and 6-methyl-2-pyridinecarbonitrile. Time of reflux was 1*h*.
The crude product was crystallized from methanol: yield 1.7 g (10%); mp 158.0-159.3 ^oC;
NMR ($CDCl_3$) δ 2.65 (s, 3H, CH_3), 7.25-7.28 (m, 2H, 2-pyr H-5 and 2-pyr H-4'), 7.56-8.00 (m, 5H), 8.22-8.47 (br, 1H,NH), 8.27 (d, J = 8.0 Hz,1H, H-8), 8.42 (s, 1H, H-4), 8.66 (d, J = 7.5 Hz, 1H, 2-pyr H-5'), 8.79 (d, J = 4.5 Hz,1H, 2-pyr H-6), 9.06 (m, 1H, 2-pyr H-3), 11.02 (br, 1H, NH);
IR (KBr,cm^{-1}) 3430(NH), 2980(CH), 1620, 1585, 1565, 1550(C=C,C=N), 1480, 1450, 1425, 1400, 1380, 1365, 1345, 1145, 1080, 1015, 990, 930, 895, 885, 860, 810, 615, 590, 570, 530;
MS, m/e 339.1455 (M^+), 339.1484 ($C_{21}H_{17}N_5$). Anal.($C_{21}H_{17}N_5$) C, H, N.

N-[3-(2-pyridyl)isoquinolin-1-yl)-6-methyl-4-pyridinecarboxamidine (3g)
This compound was synthesized and isolated in the same way as compound **3f**:
yield 15.3 g (90%); mp 206.6-209.2 ^oC;
NMR ($CDCl_3$) δ 2.70 (s, 3H, CH_3), 7.30 (m, 1H, 2-pyr H-5), 7.61-7.96 (m, 6.5H), 8.18 (d, J = 8.0 Hz, 1H, H-8), 8.42 (s, 1H, H-4), 8.68 (d, J = 6.3 Hz, 1H, 4-pyr H-5), 8.75 (d, J = 4.5 Hz, 1H, 2-pyr H-6), 8.94 (m, 1H, 2-pyr H-3);

IR (KBr,cm^{-1}) 3340(NH), 3040(CH), 1615, 1605, 1580, 1565, 1540, 1510(C=C,C=N), 1480, 1465, 1420, 1380, 1350, 1330, 1145, 1090, 1040, 925, 895, 875, 845, 780, 750, 725, 700, 615, 585, 570, 545, 525. Anal.($C_{21}H_{17}N_5$) C, H, N.

N-(2-pyridyl)-2-pyridinecarboxamidine (4)

A solution of 0.1 mol 2-aminopyridine in 100 mL anhydrous THF was stirred under a nitrogen atmosphere and cooled to -10°C. Subsequently 62.5 mL 1.6 M *n*-butyllithium in hexane was added dropwise and stirring was continued for ten minutes. Subsequently 0.1 mol 2-pyridinecarbonitrile in 50 mL anhydrous THF was added and the mixture was refluxed for three hours. After cooling to room temperature the mixture was hydrolyzed by the addition of a small amount of water. The organic phase was evaporated and the pH of the remaining water layer was adjusted to 8 with a dilute bicarbonate solution. The mixture was extracted with chloroform. The combined chloroform layers were dried with anhydrous potassium carbonate and, after filtration, evaporated to dryness. The residue was crystallized from ethanol: yield 13.9 g (70%) of yellow needles; mp 105.3-106.4 °C;

NMR ($CDCl_3$) δ 6.92 (ddd, J=7.5, 5.0, 0.75 Hz, 1H, H-5'), 7.34 (m, 2H, H-3', H-4'), 7.74 (m, 2H, H-4, H-5), 7.62-8.12 (1H, NH), 8.38 (dd, J=5.0, 2.0 Hz, 1H, H-6'), 8.56 (m, 2H, H-3, H-6), 10.27 (1H, C=NH);

IR (KBr,cm^{-1}) 3350, 3180(NH), 1620, 1580, 1560(C=C,C=N), 1455, 1420, 1295, 1270, 1245, 1140, 1065, 1045, 985, 785, 745, 735, 655, 620, 555, 535, 470;

MS, m/e 198.0888 (M^+), 198.0905 ($C_{11}H_{10}N_4$). Anal. ($C_{11}H_{10}N_4$) C, H, N.

N-(5-methyl-3-phenylisoquinolin-1-yl)-2-pyridinecarboxamidine (5)

This compound was synthesized from 0.01 mol of 1-amino-5-methyl-3-phenyl- isoquinoline and 0.01 mol of 2-pyridinecarbonitrile according to the procedure described for the synthesis of amidines from 1-amino-3-(2-pyridyl)isoquinoline and electron deficient nitriles. Time of reflux was 3*h*. The precipitate that appears after hydrolysis of the reaction mixture, was filtered off and crystallized from $CH_3OH/CH_3COOC_2H_5$: yield 1.55 g (46%) of yellow needles; mp 201.3-203.0°C;

NMR ($CDCl_3$) δ 2.74 (s, 3H, CH_3), 7.35-7.67 (m, 6H), 7.81-8.14 (m, 3H),7.83 (s, 1H), 8.14-8.38 (br s, 1H, NH), 8.64 (d, J = 4.5 Hz, 1H, 2-pyr H-6), 8.83 (d, J = 8.0 Hz, 1H), 8.94 (m, 1H, 2-pyr H-3), 10.98-11.32 (br s, 1H, NH);

IR (KBr,cm^{-1}) 3360(NH), 3040(CH), 1620, 1585, 1560, 1540, 1495(C=C,C=N), 1465, 1440, 1375, 1350, 1320, 1245, 1205, 1140, 1060, 1040, 995, 930, 920, 850, 800, 765 ,740, 690, 650, 630, 580, 570;

MS, m/e 338.1539 (M^+), 338.1531($C_{22}H_{18}N_4$). Anal. ($C_{22}H_{18}N_4$) C, H, N.

Biological activity

Nutrient medium

All experiments with *Mycoplasma gallisepticum* were done in a growth medium which was a modification of the medium used by Adler [30] to cultivate this microorganism. This modified Adler medium contained 14.8 g bacteriological peptone, 5.0 g yeast extract powder, 8.16 g D-glucose.H_2O, 3.7 g NaCl, 1.79 g $Na_2HPO_4.2H_2O$, 21 mg phenol red (pH range 6.8 - 8.4), 150 mL heat-inactivated (56°C for 30 minutes) horse serum and 10^6 IU benzylpenicillin G per liter final medium.

The medium components were dissolved in twice distilled water and the pH of the solution was adjusted to 8.0 with a concentrated sodium hydroxide solution. Before adding the horse serum and the benzylpenicillin sterilization was performed by heating at 110°C for 30 minutes.

Materials

Bacteriological peptone and yeast extract powder were purchased from OXOID Limited, Basingstoke, Hampshire, England.

Sterile Donor Horse Serum was obtained from Flow Laboratories, United Kingdom.

Benzylpenicillin G was a generous gift from Gist-brocades N.V., Delft, The Netherlands.

All chemicals used were of the highest quality obtainable.

Apparatus

Optical density of growing cultures were determined at 660 nm using a Zeiss PMQ3 spectrophotometer. pH measurements were performed with a saturated calomel electrode. Test tubes were incubated in a waterbath at 37°C.

Test organism

Mycoplasma gallisepticum K514, kindly supplied by the research management of Gist-brocades N.V., was used as the test organism. *Mycoplasma gallisepticum* strains can be stored at -20°C for several months [24]. After thawing at room temperature the culture was transferred to a bottle with fresh Adler medium in such a way that the original culture was diluted ten times. The culture was incubated overnight at 37°C.

When the pH of the culture had dropped to 6.8 and the density (determined as A_{660nm}) had reached a value of 0.22, the culture was used for inoculation purposes. The remaining part was stored at -20°C.

Determination of antimycoplasmal activity

The antimycoplasmal activity of all compounds was determined in the presence or the absence of copper and expressed as the minimal inhibitory concentration (MIC). In the former case the final concentration of $CuSO_4$ in the test tube was 40 μM. Tylosin and compound **1** were included as controls in every test. All compounds were dissolved in dimethylsulfoxide whereas tylosin was dissolved in water. It was established that DMSO in the final concentration in the Adler medium (1.25%) has no effect on Mycoplasmal growth. Serial two fold dilutions (in duplicate) of test compounds were made in Adler medium. Each tube, containing 3 mL of medium, was inoculated with 1 mL of a fresh culture of *Mycoplasma gallisepticum* K514 and these mixtures were incubated at 37°C for 24 hours. Mycoplasmal growth was indicated by a change in color of the indicator present in the medium. The minimal inhibitory concentration was determined as the lowest concentration which did not cause a change in color.

Data processing

Statistical correlations were performed by using a commercial multiple linear regression program (Statworks, Cricket Software Inc., Philadelphia, USA).The figures in parentheses are the standard errors of regression coefficients.The parameters included in each equation are significant on a 1% level. For a given equation , n is the number of compounds, r is the multiple correlation coefficient, s is the standard error of estimate and F represents the value of the F-test.

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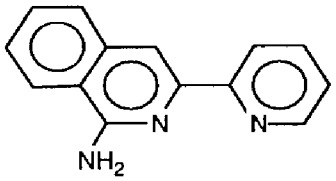
Chapter 6

SYNTHESIS AND COPPER-DEPENDENT ANTIMYCOPLASMAL ACTIVITY OF AMIDES AND AMIDINES DERIVED FROM 2-AMINO-1,10-PHENANTHROLINE*

Marcel A.H. de Zwart, Henricus M.M. Bastiaans, Henk van der Goot, and Hendrik Timmerman

Abstract A series of both aliphatic and aromatic amides and aromatic amidines derived from 2-amino-1,10-phenanthroline (**3**) according to the Topliss scheme were synthesized and subsequently tested for antimycoplasmal potency. Although the compounds themselves showed no activity, in the presence of a non-toxic copper concentration of 40 μM all compounds appeared to be very active against *M.gallisepticum*. The most active compounds were found in the amide series and show growth inhibition in the nanomolar range. These compounds are four times more active than tylosin, a macrolide antibiotic, which is used therapeutically in veterinary practice. In the presence of copper, amides derived from **3** are more active than corresponding amidines. Increased activity following derivatization of **3** may be due to the presence of a third co-ordination site for copper in the title compounds. Evaluation of biological data revealed that antimycoplasmal activity of amides derived from **3** is dependent on lipophilicity. For these amides a good linear correlation was found between antimycoplasmal activity and hydrophobic fragmental values for substituents considered. This quantitative structure-activity relationship study indicated that antimycoplasmal activity was increased upon a decrease of these hydrophobic fragmental values.



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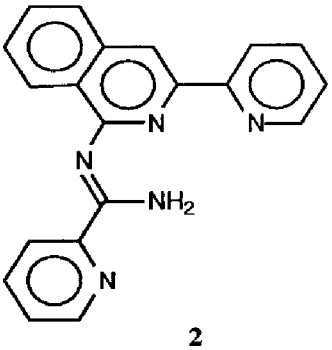

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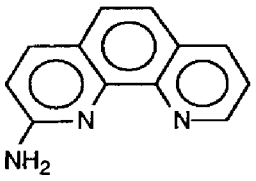


3

Introduction

It is known for many years now that compounds containing a 2,2'-bipyridyl moiety possess antimycoplasmal activity in the presence of a small non-toxic amount of copper [1].

In a proposed mechanism of action [2-4] extracellular copper is bound by the compound containing the 2,2'-bipyridyl moiety and subsequently transported across the cell membrane

* submitted for publication

into the cytosol. Once intracellular, copper itself inhibits enzymes involved in the energy providing metabolism.

In the chapters three and five investigations concerning antimycoplasmal activity of amides and amidines derived from the easy accessible 1-amino-3-(2-pyridyl)isoquinoline (**1**) have been described. For these compounds too, antimycoplasmal activity appeared to be dependent on the presence of copper. Furthermore it was found that all derivatives are more active against *Mycoplasma gallisepticum* than the parent compound, whereas the amidines are two to three times more active than the corresponding amides. It was established that the most active compound within these series viz. N-[3-(2-pyridyl)isoquinolin-1-yl]-2-pyridinecarboxamidine (**2**) is three times more active than the antimycoplasmal therapeutic tylosin.

SAR studies revealed that antimycoplasmal activity of both amides and amidines is dependent on the hydrophobic fragmental value (Σf) of the amide or amidine residue. This dependency was parabolic in nature and for both types of compounds regression analysis revealed a good correlation between antimycoplasmal activity and Σf and Σf^2.

Although we have already obtained a great increase of antimycoplasmal activity by structure optimization within a series of 1-amino-3-(2-pyridyl)isoquinoline derivatives, now we focus our attention to the corresponding 2-amino-1,10-phenanthroline (**3**) derivatives in an attempt to obtain even more active compounds.

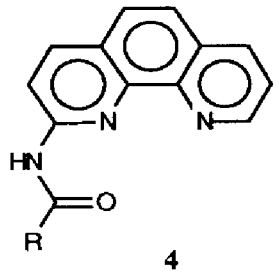
Comparison of the antimycoplasmal activity of 2-substituted 1,10-phenanthrolines with the corresponding 1-substituted 3-(2-pyridyl)isoquinolines revealed that 1,10-phenanthroline derivatives are equal or somewhat less active than the corresponding 3-(2-pyridyl)-isoquinolines [1]. However, when antimycoplasmal activity of 2-substituted 1,10-phenanthrolines is compared with antimycoplasmal activity of the corresponding 6-substituted 2,2'-bipyridyls it becomes clear that the 1,10-phenanthroline derivatives are two to four times more active than their 2,2'-bipyridyl analogues. This observation was explained by the fixed cisoid-form of the two pyridine rings in case of 1,10-phenanthroline derivatives. This cis-coplanarity of the two pyridine rings is a prerequisite for complex formation with copper and accordingly for antimycoplasmal activity.

In a structure-activity relationship study by Pijper [5] it was shown that for several compounds containing a 2,2'-bipyridyl moiety, including 2-substituted 1,10-phenanthrolines and 1-substituted 3-(2-pyridyl)isoquinolines, an optimal lipophilicity exists with regard to antimycoplasmal activity. As in general lipophilicity of 1-substituted 3-(2-pyridyl)isoquinolines, included in this study, is more close to this optimal value than lipophilicity of the analogous 2-substituted 1,10-phenanthrolines, antimycoplasmal activity of the latter is lower despite their advantageous cis-coplanarity.

Since we have demonstrated that for aromatic amides and amidines derived from

1-amino-3-(2-pyridyl)isoquinoline antimycoplasmal activity increases with decreasing lipophilicity, the lower lipophilicity of 1,10-phenanthrolines as compared to the analogous 3-(2-pyridyl)- isoquinolines may be advantageous in the case of amides and amidines derived from 2-amino-1,10-phenanthroline in regard with antimycoplasmal activity.

In this chapter we therefore report on the synthesis and antimycoplasmal activity of amides (**4**) and amidines (**5**) derived from 2-amino-1,10-phenanthroline. Structure optimization was performed according to the method proposed by Topliss [6]. Biological data are evaluated and eventually a quantitative structure-activity relationship is presented.



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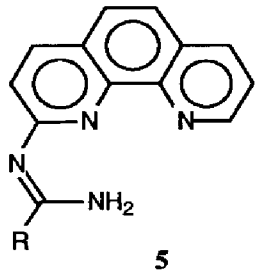

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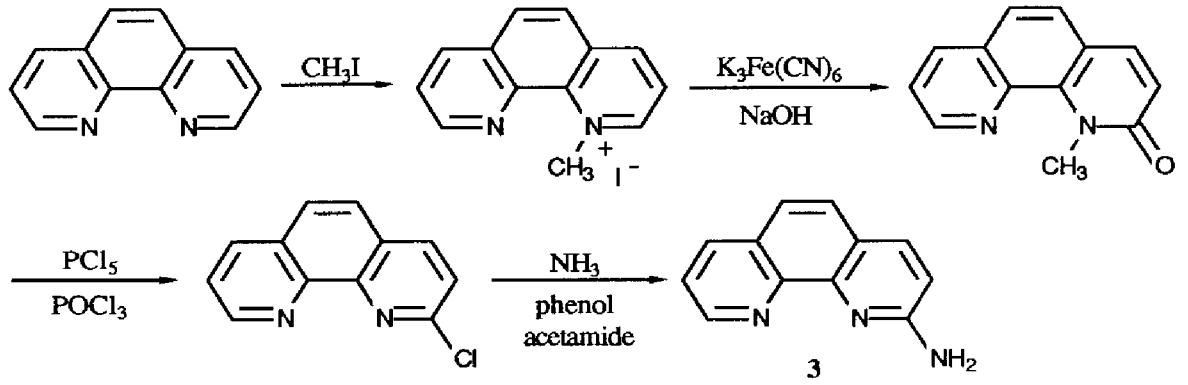
Chemistry

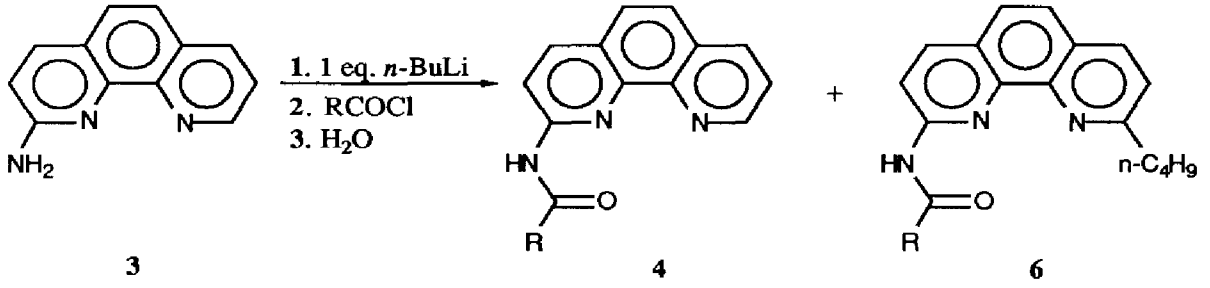
Although there are several possibilities for the synthesis of amides, we choose to prepare the desired amides **4** in the same way as we synthesized amides derived from 1-amino-3-(2-pyridyl)isoquinoline, which means by acylation of an amine with acyl chlorides [7]. The required acyl chlorides were obtained from the corresponding acids [8,9], whereas 2-amino-1,10-phenanthroline (**3**) was prepared according to literature procedures [10,11] from 1,10-phenanthroline in an over-all yield of 65% (Scheme I).

For the preparation of amides **4**, one of the amine protons is abstracted first using *n*-butyllithium as a base to increase the nucleophilicity of the amine nitrogen. Subsequently, the anion obtained is treated with one of the acyl chlorides at -15°C in anhydrous tetrahydrofuran (Scheme IIa).

Scheme I.



Scheme II a.



3 **4** **6**

By this method, referred to as method A, amides **4a-e** were obtained in moderate yields. It appeared that in some reactions considerable amounts of the 9-*n*-butyl derivative **6** were formed. Besides these 9-*n*-butyl-1,10-phenanthrolines minor amounts of diacylated 2-amino-1,10-phenanthrolines were also detected as undesired side products.

From preliminary experiments we know that diacylated 1-amino-3- (2-pyridyl)isoquinolines were not active at all against the test organism *Mycoplasma gallisepticum*. So we didn't expect any antimycoplasmal activity of these diacylated 2-amino-1,10-phenanthrolines.

In contrast with this, the butylated amides **6** seem very interesting with respect to antimycoplasmal activity, as it is known from investigations by Pijper *et al.* that di-ortho substitution of the 2,2'-bipyridyl moiety may be very advantageous for antimycoplasmal activity [1]. For this reason besides the desired amides we isolated these 9-*n*-butyl analogues too.

Although alkylation by nucleophilic substitution in pyridines by alkyllithiums is very well known, the available literature of the analogous reaction with 2,2'-bipyridyl or 1,10-phenanthroline is very scarce. Kauffmann *et al.* [12] described the alkylation of 2,2'-bipyridyl by nucleophilic substitution for the first time. Reaction of one equivalent alkyllithium with 2,2'-bipyridyl followed by hydrolysis and subsequent oxidation yielded very selectively the 6-monoalkylated compound. When an excess n-butyllithium was used, the 6,6'-dibutylated 2,2'-bipyridyl was obtained, whereas an excess methyllithium still resulted in the 6-monoalkylated compound only. Furthermore Kauffmann *et al.* [12,13] established that 2,2'-bipyridyl is more electrophilic than pyridine.

The synthesis of 6-methyl- and 6-ethyl-2,2'-bipyridyl *via* nucleophilic substitution followed by thermolytic aromatization has been described by Schmalzl *et al.* [14].

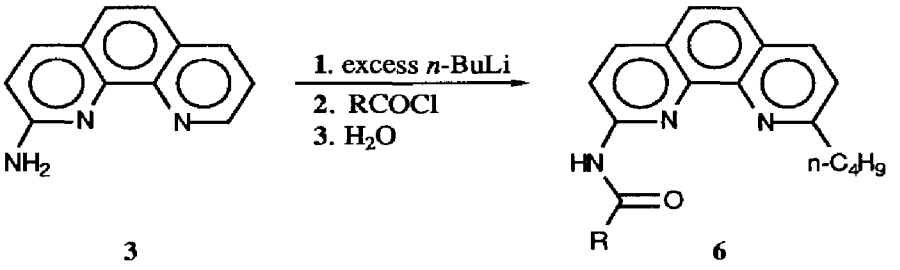
Dietrich-Buchecker *et al.* [15] found that the reaction of butyl- or phenyllithium with 1,10-phenanthroline followed by hydrolysis and rearomatization with manganese dioxide gave good yields of the 2,9-dibutyl-1,10-phenanthroline and 2,9-diphenyl-1,10-phenanthroline respectively. It was found that yields were almost entirely independent of the solvent used, but

that the nature of the dehydrogenating agent was of prime importance [15]. Nucleophilic alkylation appeared also to be applicable for the preparation of other alkyl-substituted 1,10-phenanthrolines.

Furthermore the same authors found that alkylation of 1,10-phenanthroline gave higher yields than 2,2'-bipyridyl or 2,2',6',2"-terpyridine [15]. Because of the additional ethenylene bridge the negative charge introduced by the nucleophile can apparently be stabilized by resonance more in case of 1,10-phenanthrolines. This and the fixed cis-coplanarity of the pyridyl rings, which is an advantage for the formation of the transition state in which lithium is coordinated by the two nitrogen atoms may be the reason why ortho-alkylation is observed in case of the 2-amino-1,10-phenanthrolines and not in case of the 1-amino-3-(2-pyridyl)-isoquinolines.

Initially we isolated the 9-*n*-butyl derivatives **6a-d** as a side product in the synthesis of amides **4a-e**. This was probably due to a slight excess of *n*-butyllithium which was used as a base and added as a 1.6 M solution in hexane. In an attempt to obtain these compounds as the main reaction product, we performed the same reaction using an excess of *n*-butyllithium (Scheme IIb).

Scheme II b.

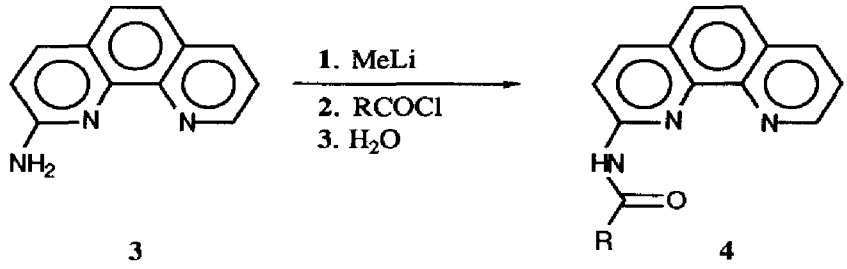


3 **6**

As expected, the 9-*n*-butylated amides **6a** and **6b** were the main reaction products and these compounds could be obtained by this method in satisfying yields (30-35%). Due to the formation of considerable amounts of side products in the synthesis of amides of 2-amino-1,10-phenanthroline by the method used, we tried to improve the synthesis of these compounds by using another base viz. methyllithium (Scheme IIc). As mentioned before, use of an excess methyllithium in alkylation of 2,2'-bipyridyl still resulted only in the mono-alkylated product [12]. Accordingly, use of methyllithium instead of *n*-butyllithium in the synthesis of amides of 2-amino-1,10-phenanthroline should give rise to less alkylation because of the decreased nucleophilic power of the base used.

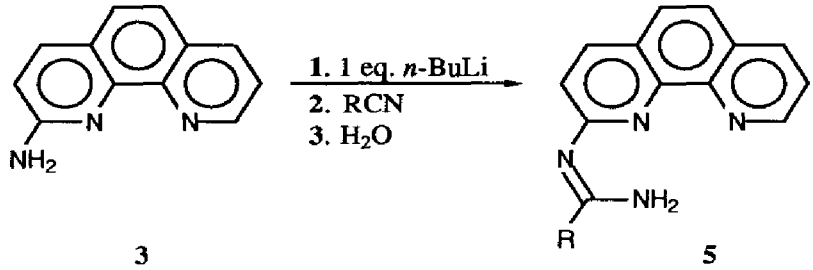
Synthesis of amides **4a-c** of 2-amino-1,10-phenanthroline by this method, referred to as method B, resulted in yields two to three times higher as compared to method A. Hence, method B indeed proved to be a good method for the synthesis of amides of 2-amino-1,10-phenanthroline and should be preferred to method A.

Scheme II c.



3 4

Scheme III.



3 5

Since not only amides of 1-amino-3-(2-pyridyl)isoquinoline but also the corresponding amidines showed antimycoplasmal activity in the presence of copper, we also synthesized some amidines of 2-amino-1,10-phenanthroline. The easiest way to obtain these kind of compounds consists of the reaction of an amine viz. 2-amino-1,10-phenanthroline with an electron-deficient nitrile (Scheme III) [16-18].

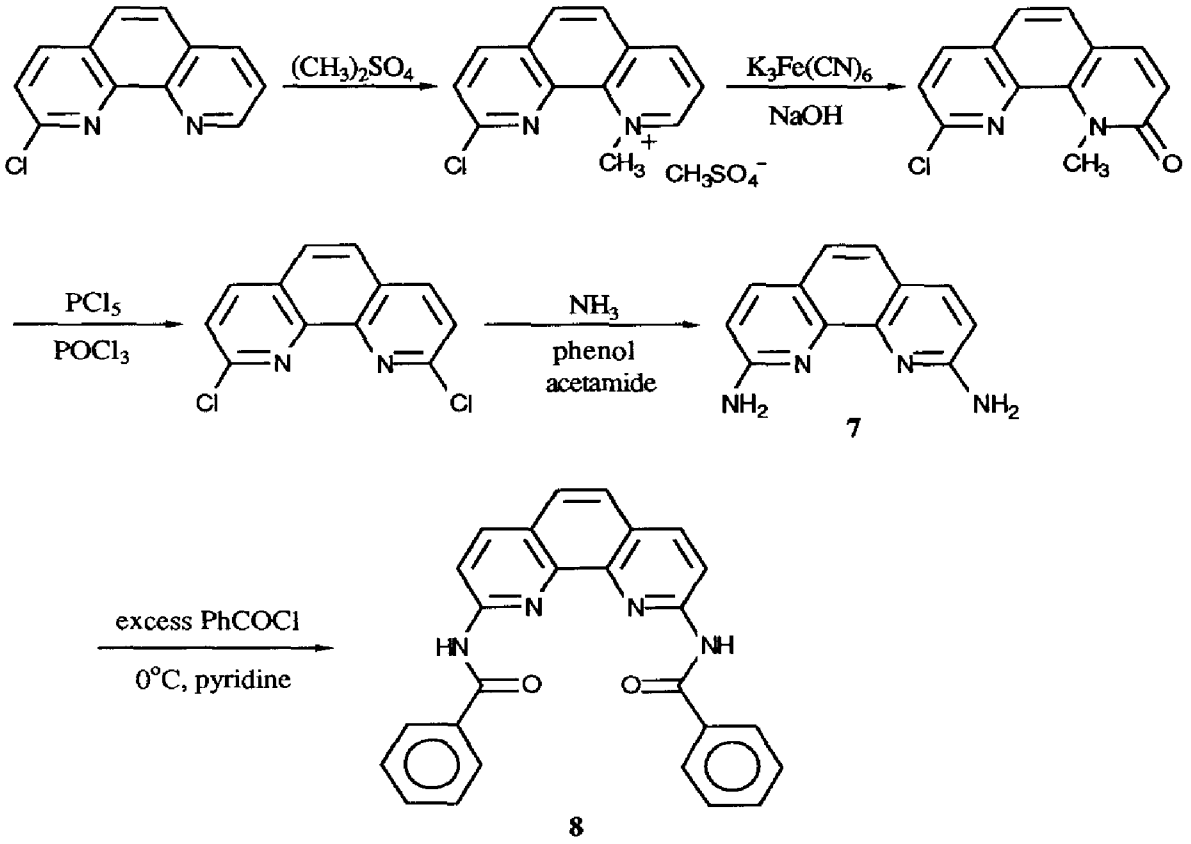
By this method amidines **5a-c** were synthesized and isolated in rather low yields. Although no alkylated product could be isolated a high number of unidentified side products were formed during this reaction. As for the amides, yields might expected to be improved by using methyllithium as a base.

As is generally known, 1,10-phenanthrolines form hydrates very easily because of the cis-coplanarity of the two pyridine rings. Also the title compounds were isolated as hydrates. Due to the complexation with a water molecule, formation of the iminol form as is described for amides of 1-amino-3-(2-pyridyl)isoquinoline is not observed in case of the amides **4a-e**.

Yamada *et al.* [19] recently described the synthesis of 6,6'-bis(benzoylamino)-2,2'-bipyridyl and its Cu (II)-complex. It was found that a 1:1 complex was formed in which the copper atom is planar co-ordinated at the two ring-nitrogen atoms and at the two amide-oxygen atoms. This supports our hypothesis that the amide or amidine moiety of the respective derivatives of 2-amino-1,10-phenanthroline may be involved in the complex formation as a third coordination site. Because of the remarkable resemblance of 2,9-bis(benzoylamino)-1,10-phenanthroline **8** with both 6,6'-bis(benzoylamino)-2,2'-bipyridyl and with the

compounds which are subject of this investigation, we synthesized compound **8** according to the procedure described by Yamada *et al.* [19] (Scheme IV) and subsequently investigated its antimycoplasmal activity. The required amine viz. 2,9-diamino-1,10-phenanthroline **7** was synthesized according to Ogawa *et al.* [13] starting from 2-chloro-1,10-phenanthroline (Scheme IV).

Scheme IV.



7

8

Biological Activity

As reported earlier, antimycoplasmal activity for a series of compounds containing a 2,2'-bipyridyl moiety is copper dependent. Amides and amidines derived from 2-amino-1,10-phenantroline **3** and 2,9-diamino-1,10-phenanthroline **7** were tested with and without addition of extra copper to the test medium. Without addition of extra copper, the copper concentration of the modified Adler medium was less than 3 μM [20]. For determination of the antimycoplasmal activity in the presence of copper, all compounds were tested in a test medium containing 40 μM $CuSO_4 \cdot 5H_2O$. This copper concentration is far below the toxic level, as the minimal inhibitory concentration was established to be 700 μM (Table I). All compounds were tested in a 3 nM to 100 μM concentration range. They were not tested for

their antimycoplasmal activity in concentrations higher than 100 μM because at this concentration they tend to precipitate when added to the growth-medium.

MIC values of the aromatic N-(1,10-phenanthrolin-2-yl)benzamides **4a-e**, their 9-n-butyl analogues **6a-d** and 2,9-bis(benzoylamino)-1,10-phenanthroline **8** are presented in Table II. MIC values of the aliphatic N-(1,10-phenanthrolin-2-yl)amides **4f,g** and the N-(1,10-phenanthrolin-2-yl)benzamidines **5a-c** are presented in Tables III and IV, respectively.

Without addition of extra copper none of the compounds under investigation showed any antimycoplasmal activity in the concentration range tested, i.e. MIC's > 100 μM. However, as was shown for other 2,2'-bipyridyl containing compounds, these compounds are very active against *M.gallisepticum* K514 in the presence of a non-toxic amount of copper. Addition of 40 μM of copper to the test medium resulted in a tremendous increase of antimycoplasmal activity.

Both amides and amidines are more active than the parent compound 2-amino-1,10-phenanthroline. In fact the most active compounds **4a,b,d** are 4 times more active on molar base than the reference compound tylosin, an antimycoplasmal drug used in veterinary practice for treatment of mycoplasmal infections.

Furthermore, amides derived from 2-amino-1,10-phenantroline **4a,d,e** are 2-8 times more active than the corresponding amidines **5a-c**. This is in contrast with what had been found in the chapters three and five for a series of amides and amidines derived from 1-amino-3-(2-pyridyl)isoquinoline, the amidines showing a 2-3 times higher activity than the corresponding amides.

Although amidines of 2-amino-1,10-phenanthroline appear to be fairly active against *M.gallisepticum* in the presence of copper, we focused our attention to the corresponding amides because of their considerable higher antimycoplasmal activity.

Structure-activity relationships

As can be seen from the MIC values reported in table II, III and IV, amides and amidines derived from 2-amino-1,10-phenanthroline themselves are not active against *M. gallisepticum*. However, addition of 40 μM copper increases their activity approximately 70 to 4000 times. Therefore it is clear that these compounds too act *via* their copper complexes as do other 2,2'-bipyridyl analogues.

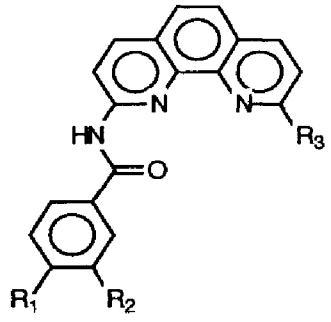
The increase of activity of the amides and amidines compared to that of their parent compound 2-amino-1,10-phenanthroline (**3**) might be attributed to the presence of a third coordination site for the copper atom in the amide and amidines derivates of **3**. This feature was already discussed in the previous chapters. Further indication for the occurrence of this third

Table I. MIC values[a] (mM) against *M.gallisepticum* K514 in a Modified Adler Medium at 37°C.

Compd	without extra copper	extra copper added[b]
$CuSO_4.5H_2O$	700	-
tylosin	0.1	0.1
3	> 100	3.1
7	> 100	0.8

[a] Number of determinations of MIC values is four. [b] 40μM $CuSO_4$.

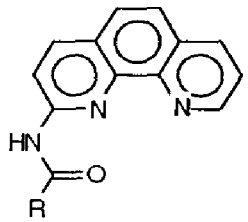
Table II. MIC values[a] (μM) against *M.gallisepticum* K514 in a Modified Adler Medium at 37°C.



Compd	R_1	R_2	R_3	without extra copper	extra copper added[b]
4a	H	H	H	> 100	0.023
4b	CH_3	H	H	> 100	0.023
4c	OCH_3	H	H	> 100	0.046
4d	Cl	H	H	> 100	0.023
4e	Cl	Cl	H	> 100	0.095
6a[c]	H	H	n-C_4H_9	> 100	0.20
6b	CH_3	H	n-C_4H_9	> 100	0.37
6c	Cl	H	n-C_4H_9	> 100	0.75
6d	Cl	Cl	n-C_4H_9	> 100	1.50
8	H	H	C_6H_5CONH	> 100	0.050

[a] Number of determinations of MIC values is two. [b] 40 μM $CuSO_4$. [c] Compound tested as HCl-salt.

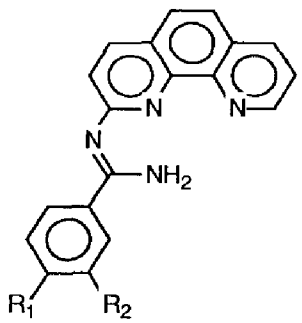
Table III. MIC values[a] (μM) against *M.gallisepticum* K514 in a Modified Adler Medium at 37 °C.



Compd	R	without extra copper	extra copper added[b]
4f	$CH(C_2H_5)C_4H_9$	> 100	0.23
4g	C_9H_{19}	> 100	0.37

[a] Number of determinations of MIC values is two. [b] 40 μM $CuSO_4$.

Table IV. MIC values[a] (μM) against *M.gallisepticum* K514 in a Modified Adler Medium at 37 °C.



Compd	R_1	R_2	without extra copper	extra copper added[b]
5a	H	H	> 100	0.2
5b	H	Cl	> 100	0.2
5c	Cl	Cl	> 100	0.2

[a] Number of determinations of MIC values is two. [b] 40 μM $CuSO_4$.

coordination site for copper is found in the work of Yamada *et al.* [19]. Their studies on the closely related 6,6'-bis(benzoylamino)-2,2'-bipyridine and its copper(II) complex revealed that 6,6'-bis(benzoylamino)-2,2'-bipyridine acts as a tetradendate copper ligand, where both amide residues are deprotonated to form a neutral complex. It is very likely that the 2,9-bis(benzoylamino)-1,10-phenanthroline also act as a tetradentate copper ligand, being able to neutralize the positive charge of the copper atom by the deprotonated amide residues. Formation of this neutral, lipophilic copper complex may be responsible for the remarkable increase of antimycoplasmal activity of 2,9-bis(benzoylamino)-1,10-phenanthroline as compared to the parent compound 2,9-diamino-1,10-phenanthroline.

Previous investigations in the chapters three and five on amide and amidines derived from 1-amino-3-(2-pyridyl)isoquinoline showed that antimycoplasmal activity was dependent on the hydrophobic fragmental value of the amide or of the amidine residue, respectively. Therefore antimycoplasmal activity of the closely related amides derived from 2-amino-1,10-phenanthroline (**3**) is likely to be predominated by the lipophilicity of the amide residue.

All amides synthesized, both aliphatic and aromatic, exist as the amide tautomer, since spectral data indicate that in no case the iminol tautomer was present. Consequently we may consider the contribution of the lipophilicity of the 1,10 -phenanthroline part with the amide moiety attached to it, to be constant for all compounds when structure-activity relationships are considered. So, for both quantitative and qualitative considerations of a possible structure-activity relationship we only take into account the part of the molecule that is varied within the series, viz. the substituent of the amide moiety and the substituent at position 9 of the 1,10-phenanthroline system.

Since these compounds show antimycoplasmal activity in the presence of copper only, this copper dependent activity was made subject of both qualitative and quantitative structure-activity relationship considerations, which resulted in the following findings. The activity sequence within the original Topliss series of amides derived from 2-amino-1,10-phenanthroline did not clearly show a dependency on the lipophilicity, as only the 3,4-dichlorobenzamide (**4e**) was significantly less active than the four other benzamides. Therefore we decided to synthesize some more lipophilic amides. The two long-chain aliphatic amides (**4f, g**) indeed are less active than the more hydrophilic benzamides (Table II and III), with the more lipohilic decanamide (**4g**) being less active than the 2-ethylhexanamide (**4f**).

Also in a series of 9-*n*-butylated amides **6a-d** the increase of activity is paralleled by a decrease of lipophilicity of the aromatic nucleus. This qualitative approach to a structure-activity relationship shows that the activity of the N-(1,10-phenanthrolin-2-yl)amides apparently is dependent on the contribution to lipophilicity of the amide residue and of the substituent at position 9 of 1,10-phenanthroline.

Combination of the three series, the benzamides **4a-e**, the 9-*n*-butylated benzamides **6a-d** and the aliphatic amides **4f, g**, shows no clear evidence for the existence of an optimal lipophilicity as we observed for both amides and amidines derived from 1-amino-3-(2-pyridyl)isoquinoline. Yet the equal antimycoplasmal activity of the benzamide (**4a**), the 4-methoxybenzamide (**4c**) and the 4-chlorobenzamide (**4d**) may be in accordance with the existence of such an optimal lipophilicity, taking into account that this optimum for lipophilicity is rather broad.

In a series of amides and amidines derived from 2-amino-1,10-phenanthroline (**3**) the amides are 2 to 8 times more active than their more hydrophilic amidine analogues (Table III and IV). From the limited series of amidines of 2-amino-1,10-phenanthroline a structure-activity relationship can not be deduced, since all amidines are equally active.

Although amidines are somewhat more hydrophilic than corresponding amides, amidines **5a-c** possess comparable lipophilicity as some of the most active amides, due to the contribution of the substituents in the benzamide moiety. So, lipophilicity alone can not account for the difference in antimycoplasmal activity of amidines as compared to amides. For an explanation of this difference, the influence of electronic and steric features should also be considered.

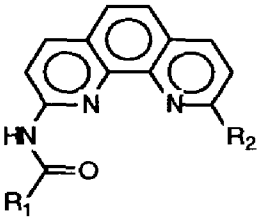
Due to the limited number of amidines a reliable quantitative structure-activity relationship can only be achieved for the amides **4a-g** and **6a-d**. In a quantitative approach to establish a structure-activity relationship we tried to correlate antimycoplasmal activity, expressed by MIC values, with the hydrophobic fragmental values (Σf) of the substituents, calculated according to Rekker [21]. Two parts of the molecule are varied, viz. the benzamide moiety and the substituent at position 9 of the 1,10-phenanthroline skeleton, being either a hydrogen atom or a *n*-butyl chain. The Σf used in regression analysis is the sum of the fragmental values of these two substituents: $\Sigma f = \Sigma f_{R1} + \Sigma f_{R2}$ (Table V).
According to Pijper *et al.* [5], 1x c_m has to be substracted from the hydrophobic fragmental values of alkyl substituents when present in the ortho position of 2,2'-bipyridyl and related compounds. By multiple regression analysis, the following equation is obtained:

$$-\log \text{MIC} = 8.825\ (\pm 0.228) - 0.545\ (\pm 0.061)\Sigma f \qquad (1)$$

$$n = 11 \qquad r = 0.948 \qquad s = 0.215 \qquad F = 80.612$$

So, within the lipophilicity range tested, we find a good lineair correlation between antimycoplasmal activity and Σf of the substituents considered. Antimycoplasmal activity is increased when the contribution to lipophilicity of the amide residue and of the substituent on carbon-9 of the 1,10-phenanthroline is decreased (Figure 1). Based on data available to us

Table V. Hydrophobic fragmental values[a]



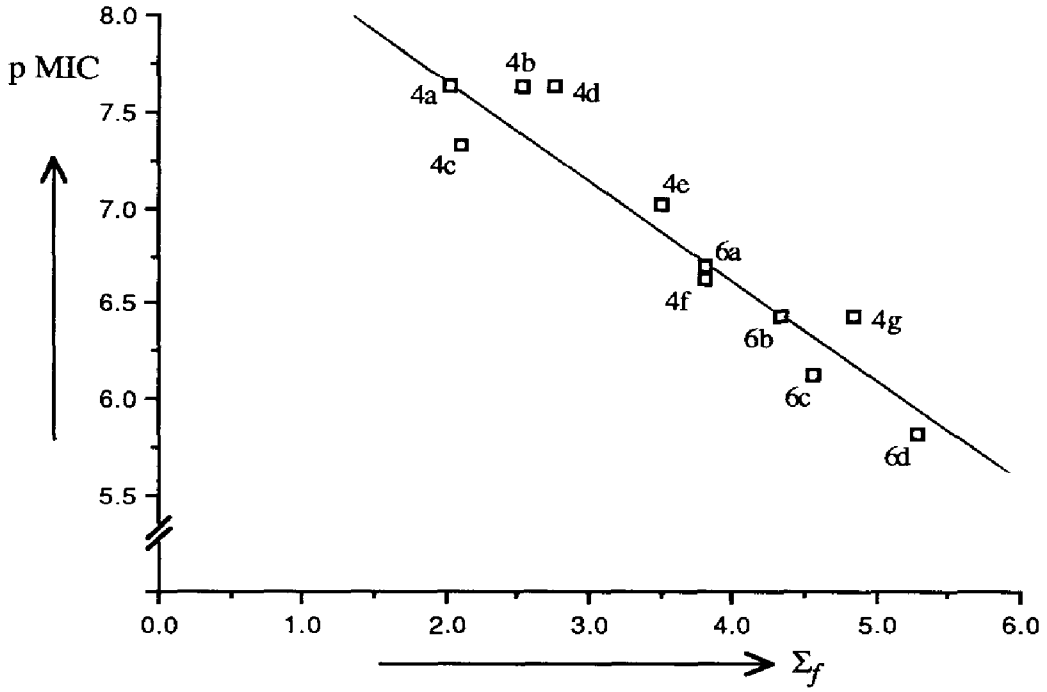
Compd	R_1	R_2	$\Sigma f_{R1,R2}$[c]	MIC (μM)$_{calcd}$[b]	MIC(μM)$_{obsd}$
4a	C_6H_5	H	2.022	0.019	0.023
4b	4-$CH_3(C_6H_4)$	H	2.541	0.036	0.023
4c	4-$OCH_3(C_6H_4)$	H	2.102	0.021	0.046
4d	4-$Cl(C_6H_4)$	H	2.764	0.049	0.023
4e	3,4-$Cl_2(C_6H_3)$	H	3.506	0.121	0.095
4f	$CH(C_2H_5)C_4H_9$	H	3.815	0.18	0.23
4g	C_9H_{19}	H	4.853	0.66	0.37
6a	C_6H_5	n-C_4H_9	3.809	0.18	0.20
6b	4-$CH_3(C_6H_4)$	n-C_4H_9	4.328	0.34	0.37
6c	4-$Cl(C_6H_4)$	n-C_4H_9	4.551	0.45	0.75
6d	3,4-$Cl_2(C_6H_3)$	n-C_4H_9	5.293	1.14	1.50

[a] See ref.[21] [b] Calculated from equation 1.

[c] $\Sigma f_{R1,R2} = f_{R1} + f_{R2}$ for compounds **4** and $\Sigma f_{R1,R2} = f_{R1} + f_{R2} - 1x\ c_m$ for compounds **6**.

now, we cannot exclude the possibility that the above linear relationship obtained, is in fact part of a relationship between antimycoplasmal activity and lipophilicity that is parabolic in nature, meaning that for these compounds too, an optimal lipophilicity with regard to antimycoplasmal activity might exist. Obviously more hydrophilic amides need to be synthesized to establish the true nature of the activity-lipophilicity relationship in a larger lipophilicity range.

Figure 1. p MIC vs Σ_f. For the identity of 4a-6d, see Table II and III.



Conclusions

Although amides and amidines derived from 2-amino-1,10-phenanthroline are not active themselves, they are very potent antimycoplasmal agents in the presence of a small amount of copper. All derivatives of 2-amino-1,10-phenanthroline are more active against *M.gallisepticum* K514 than the parent compound. It was also shown that amides are more active than corresponding amidines. In fact, the most active N-(1,10-phenanthrolin-2-yl)benzamides **4a,b,d** are 4 times more active than the antimycoplasmal therapeutic tylosin.

Antimycoplasmal activity of these compounds appeared to be dependent on their lipophilicity. For the amides, a good linear correlation was found between antimycoplasmal activity and the contribution to lipophilicity of the amide residue and of the substituent on carbon-9 of the 1,10-phenanthroline skeleton. From this relationship it was clear that antimycoplasmal activity increases upon a decrease of the hydrophobic fragmental value of these substituents.

Experimental Section

Chemistry

Melting points were determined using a Mettler FP5/FP52 apparatus.
NMR spectra were recorded on a Bruker WH-90 90 MHz spectrophotometer at 21°C. Chemical shifts are expressed in ppm relative to tetramethylsilane. Infrared spectra were recorded on a Perkin-Elmer 580B spectrophotometer. Recording of mass spectra and peak matching were performed with a Varian CH 5 DI and a Finnigan MAT 90 mass spectrometer.
All starting materials were commercially available and of the highest purity obtainable. Acyl chlorides were prepared from the corresponding carboxylic acids by standard methods [8, 9], and distilled prior to use in the acylation reaction. 3,4-Dichlorobenzonitrile was synthesized from the corresponding aldehyde [22]. 2-Amino-1,10-phenanthroline and 2,9-diamino-1,10-phenanthroline were prepared according to literature procedures starting from 1,10-phenanthroline [10, 11] (Schemes I and IV) and obtained in overall yields of 65% and 28% respectively. Melting points and spectral data were in accordance with literature data. Water content of the compounds was not only determined by titration but also by a thermogravimetric method. Results obtained were in agreement with elemental analysis data. Analytical results for compounds indicated by the molecular formula were within ± 0.4% of the theoretical values.

Synthesis

General procedure for the synthesis of amides from 2-amino-1,10-phenanthroline. Method A. (4a-e, 6b-d)

In a thoroughly dried three-necked flask, 0.02 mol of 2-amino-1,10-phenanthroline·HCl in 40 mL of anhydrous THF was stirred under nitrogen and cooled to -15°C. Subsequently 25 mL 1.6 M *n*-butyllithium in hexane was added dropwise, while maintaining the temperature at -15°C. When the addition was complete, stirring was continued for 1 *h*. Then 0.02 mol of freshly distilled acyl chloride in 10 mL of anhydrous THF was added and stirring was continued for another hour. The ice-bath was removed and when the mixture had reached room temperature it was hydrolyzed by the addition of a small amount of water. After evaporation of the organic solvent, the pH of the reaction mixture was adjusted to 8 with a dilute sodium bicarbonate solution and the water layer was extracted twice with chloroform. The combined chloroform layers were dried with anhydrous potassium carbonate, and, after filtration evaporated to dryness.

Improved general procedure for the synthesis of amides from 2-amino-1,10-phenanthroline. Method B. (4a-c,f,g)

In this procedure methyllithium was used as a base instead of *n*-butyllithium. A suspension of 0.01 mol

2-amino-1,10-phenanthroline·HCl in 40 mL of anhydrous THF was stirred under a nitrogen atmosphere and cooled to 5 °C. Subsequently, 18.8 mL of 1.6 M methyllithium in diethyl ether was added dropwise, and stirring was continued for 1.5 *h*. Then the reaction mixture was cooled to -15 °C and 0.01 mol of freshly distilled acyl chloride in 10 mL of anhydrous THF was added dropwise. While the temperature was kept at -15 °C, stirring was continued for 1.5 *h*. The ice-bath was removed, and when the mixture had reached room temperature, it was hydrolyzed with water. The organic solvents were removed by evaporation and the remaining water layer was extracted with chloroform. The combined chloroform layers were washed with a diluted sodium bicarbonate solution, dried on anhydrous potassium carbonate, and, after filtration, evaporated to dryness.

N-(1,10-phenanthrolin-2-yl)benzamide (4a)

This compound was synthesized from 2-amino-1,10-phenanthroline and benzoyl chloride according to method A. The crude reaction mixture was crystallized twice from $CH_3OH/CH_3COOC_2H_5$. The crystals obtained first, appeared to be the diacylated, C_9-butylated product. The filtrate was concentrated and the residue crystallized from $CH_3OH/CH_3COOC_2H_5$: yield 1.73 g (27%) of the monohydrate.

Prepared by method B. The product obtained as the monohydrate was crystallized twice from CH_3OH: yield 1.71 g (54%) of white needles; mp 73.7-74.7 °C;

NMR ($CDCl_3$) δ 7.44-7.60 (m, 3 H, ϕ H-3, ϕ H-4, ϕ H-5), 7.60 (dd, J = 8.1, 4.3 Hz, 1 H, H-8), 7.67 and 7.77 (AB system, J_{ab} = 9.0 Hz, 2 H, H-5, H-6), 7.96-8.08 (m, 2 H, ϕ H-2, ϕ H-6), 8.22 (dd, J = 8.5, 1.8 Hz, 1 H, H-7), 8.30 (d, J = 9.0 Hz, 1 H, H-3), 8.82 (d, J = 9.0 Hz, 1 H, H-4), 9.14 (dd, J = 4.1, 1.8 Hz, 1 H, H-9), 9.45 (s (br), 1 H, NH);

IR [23] (KBr, cm^{-1}) 3380 (br) (H_2O), 3280 (sh) (NH), 3060, 3040 (CH(ar)), 1675 (s) (C=O), 1610, 1590, 1575, 1535, 1505 (s), 1480 (s) (C=C, C=N), 1440, 1420, 1390, 1340, 1325, 1310, 1270, 1255, 1190, 1140, 1130, 1085, 1030, 920, 900, 850, 840 (o.o.p CH), 825, 795, 770, 740, 720, 700, 665, 630, 550, 510, 415, 290;

MS, *m/e* 299.11 ± 0.01 (M^+), 299.1059 ($C_{19}H_{13}N_3O$). Anal. ($C_{19}H_{13}N_3O·H_2O$) C, H, N.

4-Methyl-N-(1,10-phenanthrolin-2-yl)benzamide (4b)

This compound was synthesized from 2-amino-1,10-phenanthroline and 4-methylbenzoyl chloride according to method A. The crude reaction mixture, containing **4b** and its 9-*n*-butylated analogue **6b** was purified via column chromatography using silica gel 60 H with diethyl ether saturated with ammonia as eluent. Fractions with the same components were pooled, evaporated to dryness and crystallized from $CH_3OH/CH_3COOC_2H_5$: yield 0.64 g (10%) of N-[1,10-phenanthrolin-2-yl]-4-methylbenzamide·$^1/_2H_2O$ and 0.64 g (10%) of N-[1,10-phenanthrolin- 2-yl]-4-methylbenzamide·H_2O; mp (hemi hydrate) 109.4-110.0 °C, (mono hydrate) 75.3-76.2 °C;

Prepared by method B. The crude reaction mixture was crystallized from CH_3OH: yield 1.6 g (48%) of the monohydrate of **4b**; mp 75.0-76.1 °C; NMR ($CHCl_3$) δ 2.46 (s, 3 H, CH_3), 7.25 and 7.98 (AA'BB' system, J_{ab} = 8.6 Hz, 4 H), 7.64 (dd, J = 8.1, 4.3 Hz, 1H, H-8), 7.69 and 7.82 (AB system, J_{ab} = 9.0 Hz, 2 H, H-5,

H-6), 8.26 (dd, J = 8.5, 1.8 Hz, 1 H, H-7), 8.32 (d, J = 9.0 Hz, 1 H, H-3), 8.86 (d, J = 9.0 Hz, 1 H, H-4), 9.18 (dd, J = 4.1, 1.8 Hz, 1 H, H-9), 9.42 (s (br), 0.8 H, NH);

IR (KBr, cm^{-1}) 3430 (br) (H_2O), 3300 (br), 3140 (NH), 3040, 3020 (CH(ar)), 2920 (CH_3), 1675 (C=O), 1610, 1590, 1575, 1540, 1505 (s), 1480 (s) (C=C, C=N), 1440, 1420, 1385 (s), 1325, 1310 (s), 1265, 1250, 1190, 1135, 1120, 1090, 1080, 1020, 920, 900, 845 (s) (o.o.p CH), 825, 810, 790, 770, 745, 730, 720, 685, 645, 625, 610, 550, 510, 495, 480, 415, 260;

MS, *m/e* 313.12 ± 0.01 (M^+), 313.1215 ($C_{20}H_{15}N_3O$). Anal. (hemi hydrate) ($C_{20}H_{15}N_3O \cdot 1/2H_2O$) C, H, N.

4-Methoxy-N-(1,10-phenanthrolin-2-yl)benzamide (4c)

This compound was synthesized from 2-amino-1,10-phenanthroline and 4-methoxybenzoyl chloride according to method A. The crude reaction mixture was crystallized from $CH_3OH/CH_3COOC_2H_5$. The precipitate consisted mainly of diacylated, 9-*n*-butylated amide. The filtrate was purified via column chromatography using silica gel 60 H with diethyl ether/chloroform/triethyl amine (8:1:1) as eluent. The fractions with the same components were pooled and crystallized several times from CH_3OH or CH_3COCH_3: yield 1.05 g (15%) of the monohydrate of **4c**;

Prepared by method B. The crude reactionmixture was crystallized from CH_3OH: yield 1.56 g (45%) of the monohydrate of **4c**; mp 82.3-83.4 °C; NMR ($CHCl_3$) δ 3.91 (s, 3 H, CH_3), 7.02 and 8.02 (AA'BB' system, J_{ab}= 8.6 Hz, 4 H), 7.64 (dd, J = 8.1, 4.3 Hz, 1H, H-8), 7.69 and 7.83 (AB system, J_{ab} = 9.0 Hz, 2 H, H-5, H-6), 8.26 (dd, J = 8.5, 1.8 Hz, 1 H, H-7), 8.31 (d, J = 9.0 Hz, 1 H, H-3), 8.82 (d, J = 9.0 Hz, 1 H, H-4), 9.18 (dd, J = 4.1, 1.8 Hz, 1 H, H-9), 9.24 (s (br), 0.9 H, NH);

IR (KBr, cm^{-1}) 3580 (br) (H_2O), 3400 (br),3330 (br) (NH), 3020 (CH(ar)), 2850 (W) (OCHl), 1670 (C=O), 1605 (s), 1590, 1575, 1540, 1510, 1505 (s), 1485 (s) (C=C, C=N), 1440, 1420, 1385, 1325, 1310, 1270 (sh), 1250 (s), 1230 (sh), 1190, 1140, 1100, 1090, 1080, 1020, 920, 900, 850, 840 (o.o.p. CH), 800, 780, 760, 740, 720, 705, 680, 645, 615, 550, 445, 420, 305, 290;

MS, *m/e* 329.120 ± 0.01 (M^+), 329.1164 ($C_{20}H_{14}N_3O_2$). Anal. ($C_{20}H_{14}N_3O_2 \cdot H_2O$) C, H, N.

4-Chloro-N-(1,10-phenanthrolin-2-yl)benzamide (4d)

This compound was synthesized from 2-amino-1,10-phenanthroline and 4-chlorobenzoyl chloride according to method A. The crude reaction mixture was crystallized from $CH_3OH/CH_3COOC_2H_5$.The white crystals obtained first, appeared to be the 9-*n*-butylated amide **6c**. After filtration of these crystals the mother liquor was concentrated and the precipitate was recrystallized from $CH_3OH/CH_3COOC_2H_5$: yield 1.79 g (26%) of the monohydrate of **4d**; mp 96.1- 97.1°C;

NMR ($CHCl_3$) δ 7.52 and 8.02 (AA'BB' system, J_{ab}= 8.6 Hz, 4 H), 7.66 (dd, J = 8.1, 4.3 Hz, 1H, H-8), 7.74 and 7.85 (AB system, J_{ab} = 9.0 Hz, 2 H, H-5, H-6), 8.30 (dd, J = 8.5, 1.8 Hz, 1 H, H-7), 8.36 (d, J = 9.0 Hz, 1 H, H-3), 8.81 (d, J = 9.0 Hz, 1 H, H-4), 9.18 (dd, J = 4.1, 1.8 Hz, 1 H, H-9);

IR (KBr, cm^{-1}) 3370 (br) (H_2O), 3270 (sh) (NH), 3050, 3020 (CH(ar)), 1675 (C=O), 1610, 1595, 1575, 1540, 1505 (s), 1480 (s) (C=C, C=N), 14440,1420, 1400, 1385, 1320, 1305, 1270, 1250, 1185, 1140, 1130, 1110, 1095, 1080, 1010, 920, 900, 850 (s) (o.o.p.CH), 825, 800, 775, 750, 740, 720, 630, 540, 405, 330;
MS, *m/e* 333.055 ± 0.01 (M^+), 333.0669 ($C_{19}H_{12}Cl\,N_3O$, ^{35}Cl). Anal. ($C_{19}H_{12}ClN_3O{\cdot}H_2O$) C, H, N, Cl.

3,4-Dichloro-N-(1,10-phenanthrolin-2-yl)benzamide (4e)

This compound was synthesized from 2-amino-1,10-phenanthroline and 3,4-dichlorobenzoyl chloride according to method A. The crude reaction mixture was crystallized from $CH_3OH/CH_3COOC_2H_5$. The precipitate consisted of two compounds, **4e** and **6d**, with **6d** as the major component. The filtrate was concentrated and the precipitate consisted for the most part of **4e** and contained only a small amount of **6d**. Both crops were purified via column chromatography using silica gel 60 H with diethyl ether/chloroform/triethyl amine (8:1:1) as eluent: yield 0.88 g (11%) of monohydrate of **4e**; mp 246.1-247.4 °C;
NMR ($CHCl_3$) δ 7.62 (d, J = 8.1 Hz, 1 H, ϕ H-5), 7.65 (dd, J = 8.1, 4.3 Hz, 1H, H-8), 7.74 and 7.84 (AB system, J_{ab}= 9.0 Hz, 2 H, H-5, H-6), 7.91 (dd, J = 8.1, 1.8 Hz, 1H, ϕ H-6), 8.18 (d, J = 1.8 Hz, 1H, ϕ H-2), 8.30 (dd, J = 8.5, 1.8 Hz, 1 H, H-7), 8.36 (d, J = 9.0 Hz, 1 H, H-3), 8.75 (d, J = 9.0 Hz, 1 H, H-4), 9.18 (dd, J = 4.1, 1.8 Hz, 1 H, H-9);
IR (KBr, cm^{-1}) 3370 (H_2O), 3290 (sh) (NH), 3060 (CH(ar)), 1680 (C=O), 1640, 1620, 1610, 1590, 1570, 1545, 1505 (s), 1485 (C=C, C=N), 1460, 1440, 1420, 1395, 1340, 1325, 1295, 1275, 1230, 1140, 1095, 1085, 1030, 910, 900, 850 (sh), 845 (o.o.p. CH), 830, 815, 775, 735, 705, 680, 650, 625, 575, 545, 415, 400, 330;
MS, *m/e* 367.030 ± 0.01 (M^+), 367.0279 ($C_{19}H_{11}Cl_2N_3O$, ^{35}Cl). Anal. ($C_{19}H_{11}Cl\,N_3O{\cdot}H_2O$) C, H, N, Cl.

2-Ethyl-N-(1,10-phenanthrolin-2-yl)hexanamide (4f)

This compound was synthesized from 2-amino-1,10-phenanthroline and 2-ethylhexanoyl chloride according to method B. The crude product was purified via column chromatography using silica gel 60 H with diethyl ether saturated with ammonia as eluent. The fractions containing **4f** were pooled and after evaporation of the diethyl ether, the product was crystallized from petroleum ether (60-80 °C): yield 1.43 g (45%) of white needles; mp 193.5-194.3 °C;
NMR ($CDCl_3$) δ 0.84-1.98 (m, 14 H, C_2H_5, C_4H_9), 1.98-2.38 (m, 1 H, CH), 7.63 (dd, J = 8.1, 4.3 Hz, 1 H, H-8), 7..66 and 7.78 (AB system, J_{ab} = 9.0 Hz, 2 H, H-5, H-6), 8.24 (dd, J = 8.5, 1.8 Hz, 1 H, H-7), 8.25 (d, J = 9.0 Hz, 1 H, H-3), 8.65 (s (br), 0.8 H, NH), 8.76 (d, J = 9.0 Hz, 1 H, H-4), 9.18 (dd, J = 4.1, 1.8 Hz, 1 H, H-9);
IR (KBr, cm^{-1}) 3195, 3120, 3095 (NH), 3025, 3010 (CH(ar)), 2960, 2935, 2875, 2860 (CH(al)), 1685 (s) (C=O), 1605, 1590, 1570, 1530, 1505 (s), 1480 (s) (C=C, C=N), 1440, 1385, 1330,, 1305 (s), 1175, 1140,

1100, 1080, 850 (o.o.p. CH), 830, 795, 770, 740, 720, 655, 625, 415;
MS, *m/e* 321.187 ± 0.008 (M^+), 321.1841 ($C_{20}H_{23}N_3O$). Anal. ($C_{20}H_{23}N_3O$) C, H, N.

N-(1,10-phenanthrolin-2-yl)decanamide (4g)

This compound was synthesized from 2-amino-1,10-phenanthroline and decanoyl chloride according to method B.The product was obtained in the same way as compound **4f** and was isolated as the monohydrate: yield 1.32 g (38%); mp 72.2-73.3 °C;
NMR ($CDCl_3$) δ 0.76-2.44 (m, 19 H, C_9H_{19}), 7.62 (dd, J = 8.1, 4.3 Hz, 1 H, H-8), 7.66 and 7.80 (AB system, J_{ab} = 9.0 Hz, 2 H, H-5, H-6), 8.25 (dd, J = 8.5, 1.8 Hz, 1 H, H-7), 8.26 (d, J = 9.0 Hz, 1 H, H-3), 8.66 (d, J = 9.0 Hz, 1 H, H-4), 8.74 (s (br), 0.7 H, NH), 9.16 (dd, J = 4.1, 1.8 Hz, 1 H, H-9);
IR (KBr, cm^{-1}) 3380 (H_2O), 3215 (br) (NH), 3040 (CH(ar)), 2960, 2920, 2850 (CH(al)), 1700 (s) (C=O), 1645, 1610, 1570, 1540, 1505 (s), 1480 (s) (C=C, C=N), 1470, 1440, 1420, 1390, 1330, 1310, 1275, 1230, 1140, 1130, 1095, 1080, 850 (o.o.p. CH), 825, 815, 770, 740, 720, 655, 625;
MS, *m/e* 349.212 ± 0.01 (M^+), 349.2154 ($C_{22}H_{27}N_3O$). Anal. ($C_{22}H_{27}N_3O \cdot H_2O$) H, N; C: calcd, 71.90, found, 71.33.

N-(9-n-butyl-1,10-phenanthrolin-2-yl)benzamide (6a)

The following procedure can be used as a general procedure for the synthesis of C_9-butylated amides from 2-amino-1,10-phenanthroline. A suspension of 0.01 mol 2-amino-1,10-phenanthroline·HCl in 40 mL of anhydrous THF was stirred under a nitrogen atmosphere and cooled to 5 °C. Subsequently, 18.8 mL of 1.6 M *n*-butyllithium in hexane was added dropwise, and stirring was continued for 1.5 *h*. Then the reaction mixture was cooled to -15 °C and 0.01 mol of freshly distilled benzoyl chloride in 10 mL of anhydrous THF was added dropwise. While the temperature was kept at -15 °C, stirring was continued for 1.5 *h*. The ice-bath was removed, and when the mixture had reached room temperature, it was hydrolyzed with water. The organic solvents were removed by evaporation and the remaining water layer was extracted with chloroform. The combined chloroform layers were washed with a diluted sodium bicarbonate solution, dried with anhydrous potassium carbonate, and, after filtration, evaporated to dryness. The crude reaction mixture was purified via column chromatography using silica gel 60 H with diethyl ether/chloroform (7:3) saturated with ammonia as eluent. Fractions containing **6a** were pooled and evaporated to dryness, the residue was dissolved in anhydrous diethyl ether and hydrogen chloride was bubbled through the solution. The precipitate, the hydrochloride of **6a**, was dried and crystallized from C_2H_5OH: yield 1.18 g (30%); mp 201.3-201.7 °C;
NMR (salt) (DMSO-d_6) δ 0.99 (t, J = 7.2 Hz, 3 H, CH_3), 1.26-1.66 (m, 2 H, γ-CH_2), 1.78-2.11 (m, 2 H, β-CH_2), 3.26 (t, J = 7.2 Hz, 2 H, α-CH_2), 7.37-7.77 (m, 3 H, ϕ H-3, ϕ H-4, ϕ H-5), 7.98 (d, J = 8.1 Hz, 1 H, H-8), 8.16 (s, 2 H, H-5, H-6), 8.28-8.37 (m, 2 H, ϕ H-2, ϕ H-6), 8.59 (d, J= 9.0 Hz, 1 H, H-3), 8.76 (d, J = 8.5 Hz, 1 H, H-7), 8.96 (d, J = 9.0 Hz, 1 H, H-4);

NMR (free base) ($CDCl_3$) δ 0.98 (t, J = 7.2 Hz, 3H, CH_3), 1.28-2.06 (m, m, 4H, β-CH_2, γ-CH_2), 3.16 (t, J = 7.2 Hz, 2H, α-CH_2), 7.42-7.58 (m, 4H, H-8, ϕ H-3, ϕ H-4, ϕ H-5), 7.62 and 7.73 (AB system, J_{ab}= 10.8 Hz, 2H, H-5, H-6), 8.02-8.12 (m, 2H, ϕ H-2, ϕ H-6), 8.14 (d, J= 8.5 Hz, 1H, H-7), 8.38 (d, J = 9.0 Hz, 1H, H-3), 8.76 (d, J = 9.0 Hz, 1H, H-4), 9.68 (s (v br), 0.5H, NH);
IR (salt) (KBr, cm^{-1}) 3430 (br) (NH-amide-), 3060, 3020 (CH(ar)), 2960, 2930, 2875, 2855 (CH(al)), 2730 ($^+$NH-ring-), 1660 (C=O), 1640 (s), 1630 (s), 1605, 1580, 1540 (s), 1520, 1500, 1480 (C=C, C=N), 1470, 1450, 1430, 1400, 1365, 1320, 1275 (s), 1265 (s), 1245 (sh), 1230, 1220, 1200, 1185, 1160, 1095, 1075, 1030, 1000, 970, 900, 875 (s), 850 (o.o.p. CH), 815, 800, 790, 780, 725, 715, 710 (s), 700 (sh), 685, 670, 655, 635, 570, 420, 400, 370, 320;
MS, *m/e* 355.167 ± 0.007 (M^+), 355.1685 ($C_{26}H_{17}N_3O$). Anal. ($C_{23}H_{21}N_3O$·HCl) C, H, N, Cl.

N-(9-n-butyl-1,10-phenanthrolin-2-yl)-4-methylbenzamide (6b) This compound was obtained as a side product in the synthesis of **4b**. Following the chromatographic procedure described in the synthesis of **4b**, compound **6b** was crystallized from $CH_3OH/CH_3COOC_2H_5$: yield 0.19 g (3%). Compound **6b** was also synthesized and isolated in the same way as **6a**, using 4-methylbenzoyl chloride instead of benzoyl chloride: yield 1.31 g (34%) of the hydrochloride of **6b**; mp (salt) 207.0-208.0 °C; mp (free base) 165.7-166.6 °C;
NMR (salt) ($CHCl_3$) δ 1.05 (t, J = 7.2 Hz, 3 H, CH_3), 1.35-1.75 (m, 2 H, γ-CH_2), 1.89-2.22 (m, 2 H, β-CH_2), 2.46 (s, 3 H, CH_3), 3.39 (t, J = 7.2 Hz, 2 H, α-CH_2), 7.41 and 8.51 (AA'BB' system, J_{ab} = 8.6 Hz, 4 H), 7.44 (d, J = 8.5 Hz, 1 H, H-8), 7.94 (s, 2 H, H-5, H-6), 8.41 (d, J= 8.5 Hz, 1 H, H-7), 8.62 (d, J = 9.0 Hz, 1 H, H-3), 9.43 (d, J = 9.0 Hz, 1 H, H-4), 13,78 (s (br), 0.8 H, NH);
NMR (free base) ($CHCl_3$) δ 1.00 (t, J = 7.2 Hz, 3 H, CH_3), 1.26-1.70 (m, 2 H, γ-CH_2), 1.74-2.08 (m, 2 H, β-CH_2), 2.46 (s, 3 H, CH_3), 3.18 (t, J = 7.2 Hz, 2 H, α-CH_2), 7.31 and 7.97 (AA'BB' system, J_{ab} = 8.6 Hz, 4 H), 7.52 (d, J = 8.1 Hz, 1 H, H-8), 7.66 and 7.74 (AB system, J_{ab} = 10.8 Hz, 2 H, H-5, H-6), 8.15 (d, J= 8.5 Hz, 1 H, H-7), 8.28 (d, J = 9.0 Hz, 1 H, H-3), 8.77 (d, J = 9.0 Hz, 1 H, H-4), 9.42 (s (br), 0.8 H, NH);
IR (free base) (KBr, cm^{-1}) 3300 (br) (H_2O), 3230 (sh) (NH), 3050 (CH(ar)), 2960, 2930, 2860 (CH(al)), 1675 (sh) (C=O), 1660 (s), 1610, 1590, 1570, 1565, 1525, 1510, 1490 (s) (C=C, C=N), 1465, 1425, 1365, 1325, 1305 (s), 1280, 1265, 1215, 1190, 1140, 1105, 1085, 1020, 905, 880, 850 (o.o.p. CH), 835 (sh), 720, 745, 640, 610, 590, 540, 425;
MS, *m/e* 369.181 ± 0.009 (M^+), 369.1841 ($C_{24}H_{23}N_3O$). Anal. (free base) ($C_{24}H_{23}N_3O·H_2O$) C, H, N. Anal. (salt) ($C_{24}H_{23}N_3O$·HCl) C, H, N.

N-(9-n-butyl-1,10-phenanthrolin-2-yl)-4-chlorobenzamide (6c)
This compound was isolated as a side product in the synthesis of **4d**, by crystallization of the crude reaction mixture from $CH_3OH/CH_3COOC_2H_5$: yield 1.81 g (23%) of the monohydrate of **6c**; mp 186.1-186.2 °C;
NMR ($CHCl_3$) δ 0.98 (t, J = 7.2 Hz, 3 H, CH_3), 1.23-1.62 (m, 2 H, γ-CH_2), 1.64-1.97 (m, 2 H, β-CH_2), 3.04 (t, J = 7.2 Hz, 2 H, α-CH_2), 7.30 and 7.79 (AA'BB' system, J_{ab} = 8.6 Hz, 4 H), 7.44 (d, J = 8.1 Hz, 1 H,

H-8), 7.66 (d, J= 8.5 Hz, 1 H, H-7), 7.72 (s, 2 H, H-5, H-6), 8.08 (d, J = 8.6 Hz, 1 H, H-3), 8.19(d, J = 8.6 Hz, 1 H, H-4);

IR (KBr, cm^{-1}) 3370 (w), 3320 (w), 3070 (NH), 3040 (CH(ar)), 2950, 2930, 2860 (CH(al)), 1700 (s) (C=O), 1665 (s), 1620, 1610, 1585 (s), 1570 (sh), 1550, 1505, 1495 (s), 1485 (sh) (C=C, C=N), 1465, 1425, 1400, 1360, 1325 (sh), 1305 (s), 1285 (s), 1260 (s), 1245 (s), 1335 (s), 1230 (sh), 1210 (sh), 1170, 1145, 1125, 1105, 1090 (s), 1055, 1010, 970, 950, 905, 880, 865, 855 (s), 845 (s) (o.o.p. CH), 835 (sh), 795, 780, 755, 745, 725, 700, 690, 675, 635, 610, 590, 560, 540, 480, 450, 355, 275;

No MS data are available due to the occurence of a thermal reaction during heating the sample. Techniques used: CI, using isobutane as the reagent gas and EI, with direct introduction under electron impact conditions.

Anal. ($C_{23}H_{20}ClN_3O \cdot H_2O$) C, H, N, Cl.

N-[9-n-butyl-1,10-phenanthrolin-2-yl]-3,4-dichlorobenzamide (6d)

This compound was obtained as a side product in the synthesis of **4e**. Compound **6d** was isolated by the same chromatographic procedure as described in the synthesis of **4e**: yield 0.88 g (11%) of the monohydrate of **6d**; mp 149.8-150.5 °C;

NMR ($CHCl_3$) δ 0.96 (t, J = 7.2 Hz, 3 H, CH_3), 1.26-1.66 (m, 2 H, γ-CH_2), 1.72-2.05 (m, 2 H, β-CH_2), 3.13 (t, J = 7.2 Hz, 2 H, α-CH_2), 7.45 (d, J = 8.1 Hz, 1H, H-8), 7.50 (d, J = 8.1 Hz, 1 H, ϕ H-5), 7.69 (s, 2 H, H-5, H-6), 7.83 (dd, J = 8.1, 1.8 Hz, 1H, ϕ H-6), 8.11 (d, J = 8.5, 1.8 Hz, 1 H, H-7), 8.12 (d, J = 1.8 Hz, 1H, ϕ H-2), 8.26 (d, J = 9.0 Hz, 1 H, H-3), 8.56 (d, J = 9.0 Hz, 1 H, H-4);

IR (KBr, cm^{-1}) 3300 (br), 3280, 3070 (NH), 3050 (CH(ar)), 2960, 2930, 2860 (CH(al)), 1685 (C=O), 1635, 1610 (sh), 1595 (s), 1575, 1560, 1535, 1500 (sh), 1490 (s) (C=C, C=N), 1470, 1425, 1390, 1370, 1330, 1305, 1290 (sh), 1265, 1230, 1175, 1140, 110, 1030, 990, 915, 905, 885, 850 (o.o.p. CH), 820, 790, 770, 740, 710, 675, 640, 590, 460, 590, 460 (w), 420 (w);

No MS data are available due to the occurence of a thermal reaction durin heating the sample. Techniques used: DCI, using isobutane as the reagent gas and EI, with direct introduction under electron impact conditions.

Anal. ($C_{23}H_{19}Cl_2N_3O \cdot H_2O$) C, H, N, Cl.

General procedure for the synthesis of amidines from 2-amino- 1,10-phenanthroline and electron-deficient nitriles (5a-c)

A suspension of 0.02 mol 2-amino-1,10-phenanthroline·HCl in 50 mL anhydrous THF was stirred under a nitrogen atmosphere and cooled to -10 °C. Subsequently 25 mL 1.6 M *n*-butyllithium in hexane was added dropwise and stirring was continued for ten minutes.

Then 0.02 mol nitrile in a minimal amount of THF was added and, while keeping the reaction mixture at -10 °C, stirring was continued for ten minutes. When the mixture had reached room temperature, it was refluxed for 8 *h*. After cooling, the mixture was hydrolyzed by the addition of a small amount of water. The organic phase was evaporated and the remaining water layer was extracted with chloroform, after adjusting the pH to 8 with a

dilute bicarbonate solution. The combined chloroform layers were dried with anhydrous potassium bicarbonate and, after filtration, evaporated to dryness.

N-(1,10-phenanthrolin-2-yl)benzamidine (5a)

This compound was synthesized from 2-amino-1,10-phenanthroline and benzonitrile.The crude reaction mixture was purified via column chromatography using silica gel 60 H with diethyl ether saturated with ammonia as eluent. The fractions containing **5a** were pooled and after evaporation of the solvent, the product was crystallized from CH_3OH/H_2O: yield 0.23 g (4%) of the monohydrate; mp 168.3-170.1 °C;

NMR ($CDCl_3$) δ 7.35-7.62 (m, 5H, H-3, H-8, ϕ H-3, ϕ H-4, ϕ H-5), 7.60 and 7.71 (AB system, J_{ab}= 9.0 Hz, 2H, H-5, H-6), 7.88-8.05 (m, 2H, ϕ H-2, ϕ H-6), 8.13 (d, J= 8.46 Hz, 1H, H-4), 8.18 (dd, J= 8.46, 1.8 Hz, 1H, H-7), 9.06 (dd, J= 5.4, 1.8 Hz, 1H, H-9);

IR (KBr, cm^{-1}) 3370 (broad) (NH), 3050 (broad) (CH), 1620, 1585,1580,1570, 1560, 1540, 1520, 1500 (C=C, C=N), 1490,1450, 1420, 1340, 1292, 1270, 1223, 1190, 1140, 1105, 1080, 1035, 1005, 925, 850, 830, 783, 738, 703, 690, 658, 624;

MS, m/e 297.125 ± 0.01 ($M-H^+$), 297.1141($C_{19}H_{13}N_4$).Anal.($C_{19}H_{16}N_4O$) C, H, N.

4-Chloro-N-(1,10-phenanthrolin-2-yl)benzamidine (5b)

This compound was synthesized from 2-amino-1,10-phenanthroline and 4-chlorobenzonitrile. A little petroleum ether (60-80°C) was poured on the crude reaction mixture to obtain yellow solid material from the brown oil. The precipitate was isolated by filtration and crystallized from $CHCl_3$/ petroleum ether (60-80°C): yield 0.16 g (2.4%)of the monohydrate; mp 84.0-86.4 °C;

NMR ($CDCl_3$) δ 7.4 and 7.9 (AA'BB' system, J_{ab}= 8.1 Hz, 4H, ϕ H), 7.44-7.63 (m, 2H, H-3, H-8), 7.61 and 7.73 (AB system, J_{ab} = 9.0 Hz, 2H, H-5, H-6), 8.12 (d, J= 8.1 Hz, 1H, H-4), 8.20 (dd, J= 8.1, 1.8 Hz, 1H, H-7), 9.02 (dd, J= 4.14, 1.8 Hz, 1H, H-9);

IR (KBr, cm^{-1}) 3400 (NH), 3060 (broad) (CH), 1620, 1585, 1530, 1505 (C=C, C=N), 1490, 1450, 1420, 1395, 1345, 1305, 1290, 1268, 1228, 1210, 1135, 1108, 1093, 1075, 1015, 927, 850, 840, 830, 787, 718, 690, 650, 630;

MS, m/e 331.080 ± 0.01 ($M-H^+$), 331.0751 ($C_{19}H_{12}N_4Cl$, ^{35}Cl). Anal. ($C_{19}H_{15}N_4ClO$) C, H, N, Cl.

3,4-Dichloro-N-(1,10-phenanthrolin-2-yl)benzamidine (5c)

This compound was synthesized from 2-amino-1,10-phenanthroline and 3,4-dichlorobenzonitrile. A little petroleum ether (60-80°C) was poured on the crude reaction mixture to obtain yellow solid material from the brown oil. After filtration, the precipitate was purified via column chromatography using silica gel 60 H with diethyl ether saturated with ammonia as eluent. The fractions containing **5c** were pooled and after evaporation of the solvent, the product was crystallized from $CHCl_3$/ petroleum ether (60-80°C):

yield 1.68 g (23%); mp 185.8-187.5 °C;
NMR ($CDCl_3$) δ 7.45-7.91 (m, 4H, H-3, H-8, ϕ H-5, ϕ H-6), 7.68 and 7.78 (AB system, J_{ab} = 9.0 Hz, 2H, H-5, H-6), 8.13 (d, J= 1.8 Hz, 1H, ϕ H-2), 8.19 (d, J=8.1 Hz, 1H, H-4), 8.23 (dd, J= 8.1, 1.8 Hz, 1H, H-7), 9.09 (dd, J= 3.96, 1.8 Hz, 1H, H-9);
IR (KBr, cm^{-1}) 3350 (broad) (NH), 3160, 3060 (broad) (CH), 1635, 1620, 1588, 1525, 1505 (C=C, C=N), 1490, 1455, 1420, 1385, 1345, 1290, 1270, 1230, 1215, 1145, 1135, 1105, 1080, 1030, 980, 930, 905, 850, 840, 825, 780, 740, 660, 630;
MS, m/e 336.051 ± 0.01 (M^+), 366.0439 ($C_{19}H_{12}N_4Cl_2$, ^{35}Cl). Anal.($C_{19}H_{12}N_4Cl_2$) C, H, N, Cl.

2,9-Bis(benzoylamino)-1,10-phenanthroline (8)

This compound was synthesized according to Yamada *et al.* [19]. To obtain a better yield the reaction time was extended from 0.15 *h* to 2 *h* and the work-up procedure was slightly changed. To a suspension of 7.9 mmol 2,9-diamino-1,10-phenanthroline in 100 mL pyridine, 90 mmol benzoyl chloride was added dropwise, while keeping the reaction mixture at 0 °C. Stirring was continued for two hours. Subsequently, 600 mL diethyl ether was added to the reaction mixture and a white/yellow precipitate was obtained. This precipitate was filtered off and after washing with cold methanol dissolved in chloroform. The chloroform was washed three times with an aqueous solution of sodium bicarbonate, dried with anhydrous potassium carbonate, and, after filtration, evaporated to dryness. The residue was crystallized twice from CH_3OH: yield 1.83 g (53%) of pale yellow needles; mp 242.8-244.1°C;
NMR (DMSO-d_6) δ 7.50-7.75 (m, 6 H, ϕ H-3, ϕ H-4, ϕ H-5), 7.96 (s, 2 H, H-5, H-6), 8.15-8.24 (m, 4 H, ϕ H-2, ϕ H-6), 8.51 and 8.53 (AA'BB' system, J_{ab}= 9.0 Hz, 4 H, H-3, H-4, H-7, H-8), 11.10 (s(br), 2 H, NH).

Biological activity

Nutrient medium

All experiments with *Mycoplasma gallisepticum* were done in a growth medium which was a modification of the medium used by Adler [24] to cultivate this microorganism. This modified Adler medium contained 14.8 g bacteriological peptone, 5.0 g yeast extract powder, 8.16 g D-glucose.H_2O, 3.7 g NaCl, 1.79 g $Na_2HPO_4.2H_2O$, 21 mg phenol red (pH range 6.8-8.4), 150 mL heat-inactivated (56°C for 30 minutes) horse serum and 10^6 IU benzylpenicillin G per liter final medium.
The medium components were dissolved in twice distilled water and the pH of the solution was adjusted to 8.0 with a concentrated sodium hydroxide solution. Before adding the horse serum and the benzylpenicillin sterilization was performed by heating at 110°C for 30 minutes.

Materials

Bacteriological peptone and yeast extract powder were purchased from OXOID Limited, Basingstoke, Hampshire, England. Sterile Donor Horse Serum was obtained from Flow Laboratories,United Kingdom. Benzylpenicillin G was a generous gift from Gist-brocades N.V., Delft, The Netherlands. All chemicals used were of the highest quality obtainable.

Apparatus

Optical density of growing cultures were determined at 660 nm using a Zeiss PMQ3 spectrophotometer. pH measurements were performed with a combined glass electrode. Test tubes were incubated in a waterbath at 37°C.

Test organism

Mycoplasma gallisepticum K514, kindly supplied by the research management of Gist-brocades N.V., Delft, The Netherlands was used as the test organism. *Mycoplasma gallisepticum* strains can be stored at -20°C for several months [25]. After thawing at room temperature the culture was transferred to a bottle with fresh Adler medium in such a way that the original culture was diluted ten times. The culture was incubated overnight at 37°C.

When the pH of the culture had dropped to 6.8 and the density (determined as A_{660nm}) had reached a value of 0.22, the culture was used for inoculation purposes. The remaining part was stored at -20°C.

Determination of antimycoplasmal activity

The antimycoplasmal activity of all compounds was determined in the presence or the absence of copper and expressed as the minimal inhibitory concentration (MIC). In the former case the final concentration of $CuSO_4$ in the test tube was 40 μM. Tylosin and compound **1** were included as controls in every test. All compounds were dissolved in dimethylsulfoxide whereas tylosin was dissolved in water. It was established that DMSO in the final concentration in the Adler medium (1.25%) has no effect on Mycoplasmal growth. Serial two fold dilutions (in duplicate) of test compounds were made in Adler medium. Each tube, containing 3 mL of medium, was inoculated with 1 mL of a fresh culture of *Mycoplasma gallisepticum* K514 and these mixtures were incubated at 37°C for 24 hours. Mycoplasmal growth was indicated by a change in color of the indicator present in the medium. The minimal inhibitory concentration was determined as the lowest concentration which did not cause a change in color.

Data processing

Statistical correlations were performed by using a commercial multiple linear regression program (Statworks, Cricket Software Inc., Philadelphia, USA).The figures in parentheses are the standard errors of regression coefficients.The parameters included in each equation are significant on a 1% level. For a given equation , n is the number of compounds, r is the multiple correlation coefficient, s is the standard error of estimate and F represents the value of the F-test.

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Chapter 7

INFLUENCE OF Co(II), Ni(II), Cu(II), Zn(II) AND Cd(II) ON GROWTH-INHIBITION AND ENZYME ACTIVITY OF *Mycoplasma gallisepticum* IN THE ABSENCE AND PRESENCE OF A 2,2'-BIPYRIDYL LIGAND*

Marcel A. H. de Zwart, Henk van der Goot and Hendrik Timmerman

Abstract In the presence of a small non-toxic amount of copper 1-amino-3-(2-pyridyl)-isoquinoline derivatives are very potent antimycoplasmal agents. This activity is strongly dependent on the presence of copper. In a proposed mechanism of action copper is transported across the cell membrane into the cell by one of the 1-amino-3-(2-pyridyl)isoquinoline derivatives. Once inside the cell copper inhibits two enzymes involved in glycolysis i.e. NADH oxidase and lactate dehydrogenase. In the present investigation the influence of other metals like cobalt, nickel, zinc and cadmium on mycoplasmal growth, NADH oxidase and lactate dehydrogenase activity is determined in the presence or absence of N-[3-(2-pyridyl)isoquinolin-1-yl]-2-pyridinecarboxamidine (**1**). It was shown that even in the presence of **1** the metals investigated are not active against *Mycoplasma gallisepticum* and are less potent enzyme inhibitors as compared to copper. Cobalt and nickel even decreased antimycoplasmal activity of **1** in the presence of relatively low copper concentrations. Also antimycoplasmal activity of copper is markedly increased by ligand **1**, although in isolated enzyme preparations inhibition of both NADH oxidase and lactate dehydrogenase by copper is decreased in the presence of this ligand. These findings are consistent with the proposed mechanism of action for this kind of antimycoplasmal agents in the presence of copper.

Introduction

The single property that distinghuishes Mollicutes from all other microorganisms most, is that they are the smallest free-living prokaryotes capable of self-reproduction [1]. Besides their small size they are characterized by lack of a rigid cell wall. The individual organisms are pleomorphic and surrounded only by a trilaminar cell membrane, which is mainly composed of lipids and proteins [2].

The test organism used in this investigation is *Mycoplasma gallisepticum*. This species belongs to the class of Mollicutes and requires cholesterol for its growth. *Mycoplasma gallisepticum* is a pathogenic *Mycoplasma* species, causing chronic respiratory disease in poultry [3]. *Mycoplasma gallisepticum* is one of the fermentative mycoplasmas in which

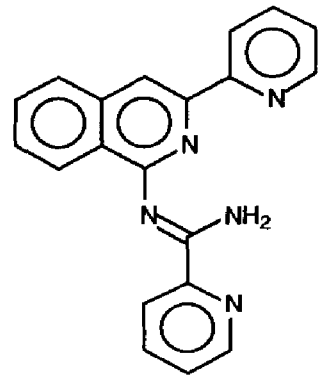
* submitted for publication

adenosine 5'-triphosphate (ATP) is formed during glycolysis [4]. Anaerobically, lactic acid is the predominant acid formed as an end product of glucose metabolism. Aerobically glucose is oxidized to acetate and carbon dioxide [5,6]. The fermentative Mollicutes have a flavin-terminated respiratory chain since they contain flavin and they lack quinones and cytochromes [7,8].

Previous investigations in our laboratory have revealed that antimycoplasmal activity of 2,2'-bipyridyl and related compounds is strongly dependent on the presence of copper ions [9,10]. In the presence of 40 μM copper one of the most potent compounds we have synthesized [11], viz. N-[3-(2-pyridyl)isoquinolin-1-yl)-2-pyridinecarboxamidine **1**, is three times more active against *Mycoplasma gallisepticum* K514 than tylosin [12], a macrolide antibiotic which is used against mycoplasmal infections in veterinary practice.

In a study on the mechanism of action of these type of compounds it was shown that they facilitate transport of copper ions across the mycoplasmal membrane through complex formation. Once inside the cell copper ions exert their main toxic effect by inhibiting enzymes involved in the glycolytic pathway i.e. NADH oxidase and lactate dehydrogenase (LDH) [13-15].

In the present investigation we have focused our attention to the influence of various metals like cobalt, nickel, zinc and cadmium on both mycoplasmal growth and enzyme activity. Furthermore the influence of **1** on antimycoplasmal activity and enzyme inhibition of these metals was investigated. The results obtained are compared with the results of corresponding experiments with copper.



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Materials and methods

Chemicals

N-[3-(2-pyridyl)isoquinolin-1-yl]-2-pyridinecarboxamidine was obtained from laboratory stock [11]. $CuSO_4.5H_2O$, $CdCl_2.21/2H_2O$, $ZnSO_4.7H_2O$, $Fe_2(SO_4)_3.nH_2O$, $FeSO_4.7H_2O$, NaOH and NaCl were obtained from J.T. Baker Chemicals, The Netherlands. $NiSO_4.6H_2O$,

$CoSO_4.7H_2O$ and $Na_2HPO_4.12H_2O$ were obtained from E. Merck AG, Darmstadt, FRG. Crystalline sodium pyruvate and crystalline bovine serum albumin were obtained from Sigma Chemicals Company, St. Louis, USA. NADH Grad II, 98% disodium salt was obtained from Boehringer, FRG. Bio-Rad protein assay dye reagent concentrate was obtained from Bio-Rad Laboratories, FRG. Water was distilled from an all glass still after deionization over a mixed-bed ion exchanger. Phosphate buffered saline (PBS) is a 10 mM sodium phosphate buffer, pH 7.2, containing 0.85% (145mM) sodium chloride.

Test organism and nutrient medium

The test organism used was *M.gallisepticum* strain K514, which was a generous gift of Gist-brocades N.V., Delft, The Netherlands. Stock cultures were maintained in growth medium at - 20°C. The nutrient medium was a modified Adler medium [16] consisting of 14.8 g bacteriological peptone (OXOID Ltd.), 5.0 g yeast extract powder (OXOID Ltd.), 8.16 g D-Glucose.H_2O, 3.7 g NaCl, 3.59 g $Na_2HPO_4.12H_2O$, 21 mg phenol red, 150 mL heat inactivated (56°C for 30 min) donor horse serum (Flow Laboratories) and 10^6 IU benzyl penicillin G (Gist-brocades N.V.) per liter final medium. The medium components were dissolved in water, and the pH of the solution was adjusted to 8.0 with a concentrated sodium hydroxide solution. Before adding the filter sterilized horse serum and penicillin, the medium was sterilized for 30 min at 110°C.

Determination of antimycoplasmal activity

Antimycoplasmal activity was determined and expressed as the minimal inhibitory concentration (MIC). When antimycoplasmal activity of N-[3-(2-pyridyl)isoquinolin-1-yl]-2-pyridinecarboxamidine (**1**) in the presence of various metals was determined the metal concentration was 40 μM. At this metal concentration no growth inhibition was observed without ligand present. Tylosin and **1** were included as controls in every test. All metals and tylosin were dissolved in water whereas **1** was dissolved in dimethylsulfoxide (DMSO). It was established that DMSO in the final concentration in the Adler medium (1.25%) has no effect on mycoplasmal growth. Serial two fold dilutions (in duplicate) of test compounds were made in Adler medium. Each tube, containing 3 mL of medium, was inoculated with 1 mL of a fresh culture of *Mycoplasma gallisepticum* K514 and these mixtures were incubated at 37°C for 24 hours. Mycoplasmal growth was indicated by a change in color of the indicator present in the medium. The minimal inhibitory concentration was determined as the lowest concentration which did not cause a change in color.

Preparation of crude cell extracts

Cells of *M.gallisepticum* were harvested in the exponential phase of growth (optical density at 660 nm = 0.2 - 0.25) by centrifugation at 8000 g, 4°C for 10 min. The cells were resuspended in a volume of 10% of the original culture in PBS and centrifuged again. After two washings the cells were resuspended in a volume of 1% of the original culture in the same buffer and cooled to 0°C. Cell extracts were prepared by sonic disruption of the cells, using an MSE ultra sonic power unit (microtip, 12 μm, low power) for six 30-sec periods alternated with cooling for 1 min. The resulting suspension was centrifuged at 40.000 g, 4°C for 30 min and the supernatant was taken as the crude cell extract. Protein content was determined using the Bio-Rad Protein Assay, with bovine serum albumin as protein standard [17]. The cell extract was divided into 500 μL portions and stored at -80°C. Different batches of cell extracts yielded comparable enzyme activities.

Determination of NADH oxidase activity

Cell extract was diluted with PBS to a protein concentration of 91 μg/mL and stored as a stock solution on ice. 800 μL of the diluted cell extract was preincubated at 25°C for 5 min with 100 μL of an aqueous solution of varying concentration of the metal under investigation, or with 100 μL water (control).When ligand was present in the assay 10 μL of a solution of an appropiate concentration of the ligand in DMSO was added. The reaction was started by addition of 100 μL of a 2.5 mM NADH solution in PBS. The oxidation of NADH at 25°C was followed spectrophotometrically at 340 nm.

Determination of lactate dehydrogenase (LDH) activity

LDH activity was determined under anaerobic conditions using a Thunberg cuvette, because under aerobic conditions, NADH oxidase competes for the common substrate NADH. Cell extract was diluted with PBS to a protein concentration of 3.6 mg/mL and stored as a stock solution on ice. 500 μL of this solution was put into the cuvette together with 1600 μL PBS, 300 μL 40 mM sodium pyruvate in PBS and 300 μL of an aqueous solution of varying concentrations of the metal under investigation or 300 μL water (control). When ligand should be present in the assay 30 μL of a solution of the ligand of an appropiate concentration in dimethylsulfoxide was added to this mixture. Consequently, 300 μL 2.5 mM NADH in PBS was placed in one loop of the cuvette top. Oxygen was removed by bubbling nitrogen through both solutions for 10 min at 25°C; it was established that this time period was sufficient to prevent the NADH oxidase reaction. The oxygen-free cuvette was closed gas tight and the reaction was started by adding the NADH solution to the mixture containing the enzyme. The oxidation of NADH was followed spectrophotometrically at 340 nm.

Apparatus

Optical density of growing cultures were determined at 660 nm with a Zeiss PMQ 3 spectrophotometer. pH measurements were performed with a saturated calomel electrode. Test tubes were incubated in a water bath at 37°C. Enzyme activity measurements were performed at 25°C on an Aminco DW-2A UV/VIS spectrophotometer at 340 nm against a control, which contained everything but NADH.

Results

Antimycoplasmal activity of Co(II), Ni(II), Cu(II), Zn(II) and Cd(II)

MIC values of the various metals were determined against *Mycoplasma gallisepticum* K514 in Adler medium at 37°C as described in Materials and Methods. The highest concentration tested of each metal was 400 µM, because compounds with a MIC value above 400 µM are considered to be inactive. MIC values of the metals are presented in table I.

Percentage growth-inhibition is calculated as follows:

$$\% \text{ growth-inhibition} = \frac{pH_x - pH_0}{pH_{100} - pH_0} \times 100\%$$

In this approach acid production by growing cells of *Mycoplasma gallisepticum* after 24 hr incubation at 37°C, expressed as a decrease of the pH of the growth-medium is taken as growth parameter.

In this equation pH_x is pH of a culture grown in the presence of a metal concentration of 400 µM, pH_0 is pH of a culture grown without inhibition and pH_{100} is pH of a 100% growth-inhibited culture, which is equal to the pH of a culture at the start of incubation.

A good linear relationship was found experimentally between the pH of a culture and its optical density at 660 nm, which is a measure of cell density.

As can be seen from table I copper is the only metal capable of complete growth-inhibition, though this complete inhibition is only seen at the highest concentration tested i.e. 400 µM. The presence of the other metals in the growth medium at a concentration of 400 µM results only in a partial reduction of microbial growth.

Antimycoplasmal activity increases in the following order: Co, Ni < Zn < Cd < Cu.

Although copper is the most active metal of the ones tested, its activity is low compared to the reference compound tylosin.

Table I. MIC values against *M.gallisepticum* K514 in Adler medium at 37°C.

Compound	MIC (μM)	% growth-inhibition[a]
$CoSO_4.7H_2O$	>400	16
$NiSO_4.6H_2O$	>40	14
$CuSO_4.5H_2O$	400	100
$ZnSO_4.7H_2O$	>400	60
$CdCl_2.21/2H_2O$	>400	68
tylosin		0.1

[a] % growth-inhibition at a metal concentration of 400 μM.

Antimycoplasmal activity of N-[3-(2-pyridyl)isoquinolin-1-yl)-2-pyridinecarboxamidine (1) in the presence of 40 μM Co(II) or Ni(II) or Cu(II) or Zn(II) or Cd(II)

MIC values of **1** are determined in the presence of 40 μM of one of the metals under investigation. Without ligand present no growth inhibition was observed at this metal concentration. The results obtained are presented in Table II.

Table II. MIC values[a] of N-[3-(2-pyridyl)isoquinolin-1-yl)2-pyridylcarboxamidine **1** in the presence of 40 μM metal

Metal	MIC (μM)
-	0.5
Co^{2+}	>4.0
Ni^{2+}	>4.0
Cu^{2+}	0.01
Zn^{2+}	0.5
Cd^{2+}	0.5

[a] MIC value of tylosin is 0.1 μM.

Without the addition of any metal to the growth medium compound **1** has a MIC value of 0.5 μM. Antimycoplasmal activity of **1** is enhanced neither by addition of cadmium nor zinc. In case of addition of nickel or cobalt to the growth medium antimycoplasmal activity of ligand **1** is even lower than without addition of any metal.

Table III. IC_{50}-values of various metals for NADH oxidase and lactate dehydrogenase in crude cell extract of *Mycoplasma gallisepticum* K514 at 25°C.

Metal	IC_{50} (mM) NADH oxidase	IC_{50} (mM) lactate dehydrogenase
Cu^{2+}	0.0005	0.003
Cd^{2+}	0.7	0.012
Zn^{2+}	1.8	0.014
Co^{2+}	2.3	3.0
Ni^{2+}	>3.0	7.5

Number of observations is two.

Antimycoplasmal activity of **1** is increased only upon the addition of copper to the growth medium. In the presence of 40 μM copper **1** is 50 times more active against *Mycoplasma gallisepticum* K514 than in the absence of copper. Antimycoplasmal activity of **1** in the presence of 40 μM metal increases in the following order: Co, Ni < Cd, Zn < Cu.

Inhibition of NADH oxidase and lactate dehydrogenase

Enzyme inhibition of various metals was investigated in crude cell extracts of *Mycoplasma gallisepticum* K514.

Influence of Co(II), Ni(II), Cu(II), Zn(II) and Cd(II) on NADH oxidase activity

Mycoplasma species contain a cytoplasmatic NADH oxidase. Klömkes *et al.* [18] described the isolation and purification of the cytoplasmatic NADH oxidase from *Mycoplasma capricolum* . The enzyme appeared to be a flavoprotein with FAD as prosthetic group and an apparent molecular mass of about 72.5 kDa. No iron, copper, manganese or molybdenium could be detected. NADH oxidase mediates the oxidation of NADH by molecular oxygen:

$$NADH + H^+ + O_2 \longrightarrow NAD^+ + H_2O_2 \qquad (1)$$

NADH oxidase activity was determined by measuring the rate of oxidation of NADH spectrophotometrically at 340 nm. The reaction rate was measured in the presence of varying metal concentrations.

The NADH oxidase assay contained: 0.25 mM NADH, 73 μg/mL protein, 10 mM PBS and

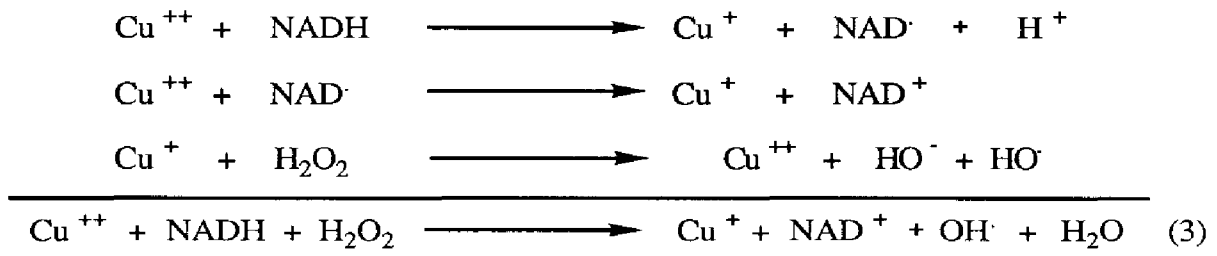
various concentrations of test compounds.
Results expressed as IC_{50}-values, obtained graphically from a % inhibition vs inhibitor concentration plot, are presented in table III. Inhibition of NADH oxidase by various metals increases in the following order: Ni < Co < Zn < Cd < Cu.
The IC_{50}-value for copper corresponds very well with data presented by Smit *et al.* [19].

Influence of Co(II), Ni(II), Cu(II), Zn(II) and Cd(II) on lactate dehydrogenase (LDH)
Neimark *et al.* [21] demonstrated the presence of a NAD-dependent L (+)-lactic dehydrogenase in *Mycoplasma gallisepticum* X-95 and in several other mycoplasma species. LDH mediates the reduction of pyruvate into lactate:

(2)

LDH activity was determined by measuring the rate of oxidation of NADH spectrophotometrically at 340 nm.
The LDH assay contained: 0.25 mM NADH, 4 mM sodium pyruvate, 600 µg/mL protein, 10 mM PBS and various concentrations of test compounds.
Results expressed as IC_{50}-values are presented in table III. Inhibition of LDH by various metals increases in the following order: Ni < Co < Zn, Cd < Cu.
The IC_{50}-value for copper corresponds very well with data presented by Smit *et al.* [19].

Influence of Fe(II) and Fe(III) on NADH oxidase activity in crude cell extract of M.gallisepticum K514
During the NADH oxidase reaction (1) hydrogen peroxyde is generated. Furthermore it was demonstrated by Rowley *et al.* [20] that addition of a Cu(II) salt to a solution of NADH in the presence of hydrogen peroxyde at pH 7.4 caused the formation of hydroxyl radicals. It was assumed that after reduction of the copper (II) ions by NADH, hydroxyl radicals are generated as a consequence of reduction of hydrogen peroxyde by copper(I) ions:



(3)

To determine whether inhibition of NADH oxidase by copper is hydroxyl radical mediated we

investigated the influence of iron(II) and iron(III) ions on this enzyme activity. It is known that hydroxyl radicals are also generated when hydrogen peroxyde is reduced by iron(II) ions (Fenton reaction)[21]:

$$Fe^{++} + H_2O_2 \longrightarrow Fe^{+++} + HO^- + HO^{\cdot} \quad (4)$$

Iron concentrations are varied from 0-300 μM. Even in the highest concentration of iron(III) ions no affection of NADH oxidase activity was observed. However, at a iron(II) ion concentration of 300 μM NADH oxidase activity was reduced with 24%. At lower concentrations no influence of iron(II) ions on NADH oxidase activity was found.

Influence of N-[3-(2-pyridyl)isoquinolin-1-yl)-2-pyridinecarboxamidine (1)in the presence of copper on enzyme activity in crude cell extract of Mycoplasma gallisepticum K514

Both NADH oxidase and LDH are only slightly inhibited by **1** in the concentration range tested. At a concentration of 30 μM, **1** inhibits both enzymatic reactions for about 10%, while copper at the same concentration almost completely (98-99%) inhibits both enzymes.

We investigated the influence of the presence of both copper and ligand **1** in a 1:2 ratio, to mimic the presence of the corresponding copper complex, on enzyme activity.

The results we obtained are presented in figure I as a %inhibition vs copper complex concentration plot. The percent inhibition was calculated from the reaction rate (V) measured at a certain copper complex concentration and the reaction rate of the uninhibited reaction (V_u) as $(1- V/V_u) \times 100\%$.

Figure I. Influence of copper in the absence or presence of N-[3-(2-pyridyl)isoquinolin-1-yl]-2-pyridine-carboxamidine (**1**) on NADH oxidase activity in crude cell extract of *M.gallisepticum* K514. Results are expressed as % inhibition of the NADH oxidase reaction caused by copper alone (▫) or copper in the presence of two equivalents **1** (○).

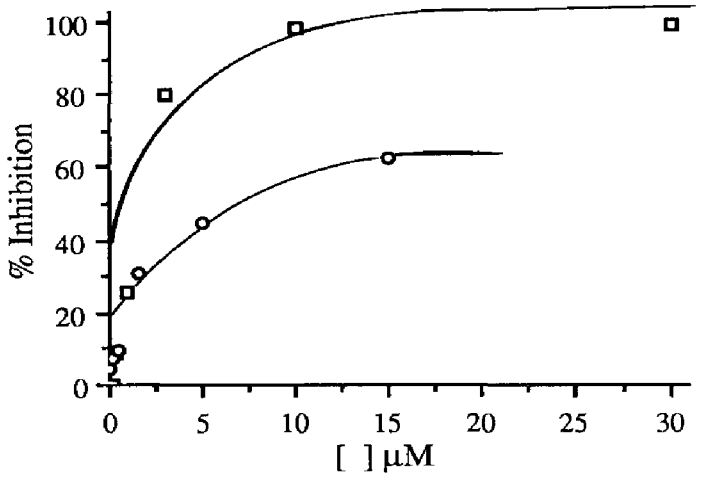
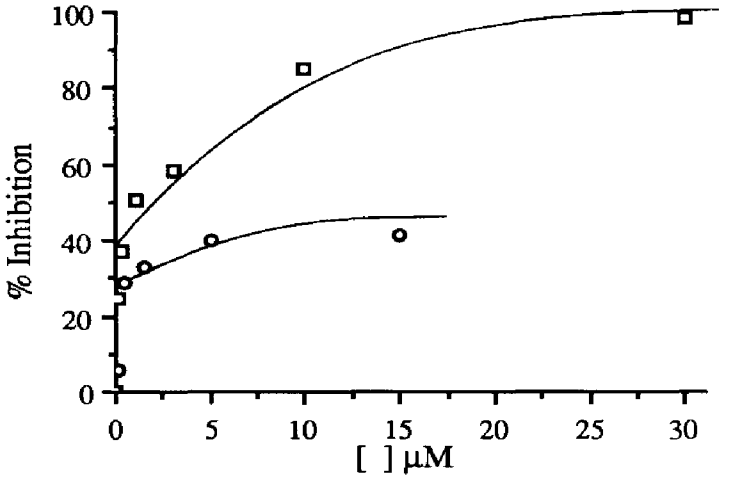


Figure II. Influence of copper in the absence or presence of N-[3-(2-pyridyl)isoquinolin-1-yl]-2-pyridine-carboxamidine (**1**) on LDH activity in crude cell extract of *M.gallisepticum* K514. Results are expressed as % inhibition of the LDH reaction caused by copper alone (□) or copper in the presence of two equivalents **1** (○).



As can be seen from this plot inhibition of NADH oxidase by the copper complex of **1** is much lower than by copper itself. The same feature is observed in case of LDH activity as can be seen from figure II. Accordingly, IC_{50}-values for the copper complex are higher than for copper itself (Table IV).

Table IV. IC_{50}-values (µM) of copper in the absence or presence of ligand **1** for NADH oxidase and LDH from crude cell extract of *Mycoplasma gallisepticum* K514.

enzyme	copper	copper/ligand (1:2)
NADH oxidase	0.5	7.2
LDH	3	> 15

Furthermore we investigated the influence of increasing amounts of ligand on the enzyme inhibition caused by copper. At a copper concentration which causes approximately 80% inhibition of enzyme activity, increasing amounts of ligand **1** were added. The highest ligand concentration tested was equal to three times the copper concentration. Results are presented as % inhibition vs ligand concentration plots in figure III and IV for NADH oxidase and LDH respectively. From these plots it is clear that inhibition of enzyme activity by copper is

decreased upon addition of ligand **1** until a 1:1 ratio of copper : ligand is reached. Further increase of ligand concentration doesn't result in a further decrease of enzyme inhibition. However, at the highest ligand concentration tested enzyme inhibition increases again.The latter may be the consequence of inhibition by the ligand itself.

Figure III. Influence of N-[3-(2-pyridyl)isoquinolin-1-yl]-2-pyridinecarboxamidine (1) on inhibition of NADH oxidase by copper in crude cell extract of *M.gallisepticum* K514 in the presence of 3 μM copper.

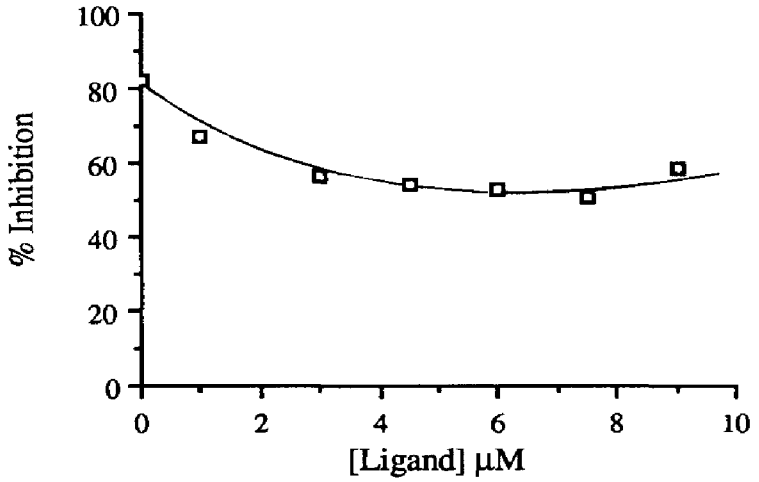
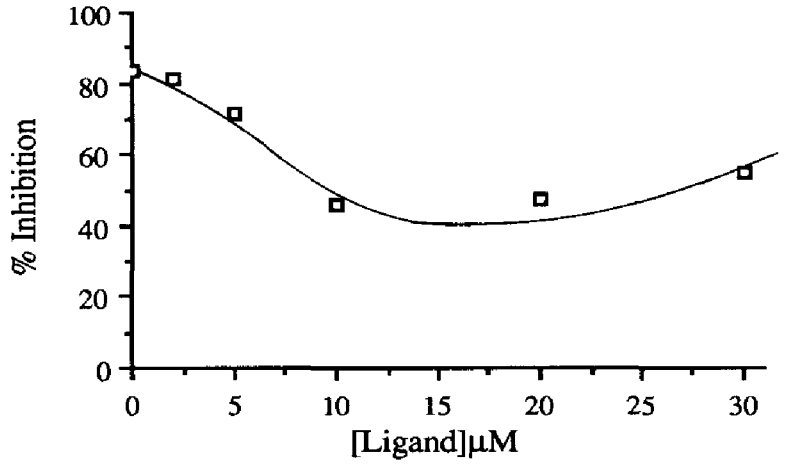


Figure IV. Influence of N-[3-(2-pyridyl)isoquinolin-1-yl]-2-pyridinecarboxamidine (1) on inhibition of LDH by copper in crude cell extract of *M.gallisepticum* K514 in the presence of 10 μM copper.



Discussion

After the discovery of antimycoplasmal activity of 1-amino-3-(2-pyridyl)isoquinolines it was established that this activity was strongly dependent on the presence of copper [9,10]. The

same feature holds true for derivatives of 1-amino-3-(2-pyridyl)isoquinoline like N-[3-(2-pyridyl)- isoquinolin-1-yl]-2-pyridinecarboxamidine (**1**) [11]. Although copper itself hardly shows any antimycoplasmal activity, compound **1** is very active in the presence of 40 μM copper against *M. gallisepticum* K514 (Table II). This striking dependency on copper raised the question whether other metals are also capable of growth inhibition of *M. gallisepticum* K514.

Our choice which metals should be investigated was led by the prerequisite that the metal should be capable of complex formation with ortho substituted 2,2'-bipyridyl type ligands. The metals investigated appeared to have no antimycoplasmal properties (table I). Minimal inhibitory concentration for all metals was greater than 100 μg/mL. This is not at all surprising because copper itself is not very active against *M. gallisepticum* K514. At the highest concentration tested these metals showed only partial growth-inhibition increasing in the following order: Co, Ni < Zn < Cd < Cu.

However, it is more interesting to investigate antimycoplasmal activity of these metals in the presence of **1**.

As ligand we have chosen N-[3-(2-pyridyl)isoquinolin-1-yl)-2-pyridinecarboxamidine (**1**), which in the presence of 40μM copper is the most active ligand derived of 1-amino-3-(2-pyridyl)isoquinoline [11,23].

In the absence of copper this compound also shows some antimycoplasmal activity (table II). This activity is ascribed to the presence of a small quantity of copper of about 3 μM [7] in the growth-medium used. In the presence of 40μM cadmium or zinc antimycoplasmal activity of **1** is not increased. In the presence of 40μM cobalt or nickel antimycoplasmal activity of **1** is even decreased. Only the presence of 40μM copper results in a considerable increase of antimycoplasmal activity of **1**. It follows that antimycoplasmal activity of 2,2'-bipyridyl related compounds is very specifically dependent on copper.

From table III it is clear that inhibition of both NADH oxidase and lactatedehydrogenase also is very specific for copper. In case of NADH oxidase IC_{50}-values of the metals investigated are at least three orders of magnitude higher than the IC_{50}-value of copper. For lactate dehydrogenase the difference in enzyme inhibiting capabality is not so extreme. Whereas cobalt and nickel inhibit LDH only in very high concentrations (mM range), cadmium and zinc are only four to five times less potent than copper.

Comparison of the antimycoplasmal activity of the metals under investigation with their enzyme inhibiting properties leads to the following considerations. Although cadmium and zinc show considerable LDH inhibitory properties they possess no antimycoplasmal activity in the presence of **1**. Cobalt and nickel show only enzyme inhibition to a small extent and they

decrease antimycoplasmal activity of ligand **1**. Comparison of the stability constants of complexes formed from the metals under investigation and 2,2'-bipyridyl [24,25] reveals that the affinity of this ligand for cadmium and zinc is considerable smaller than for nickel, cobalt and copper. Assuming that this difference also exists for complexes of these metals and ligand **1** we can explain that although cadmium and zinc are fairly good inhibitors of LDH they don't possess antimycoplasmal activity in the presence of **1**. Because of the poor complexing ability of compound **1** for zinc and cadmium these metals are more likely bound to growth-medium constituents. Therefore they will hardly penetrate into the cell and consequently they can not exert any enzyme inhibiting action.
To the contrary, compound **1** has comparable affinity for cobalt, nickel and copper. So, when cobalt or nickel is present in the growth-medium at a concentration of 40 μM, ligand **1** will bind these metals more likely than copper which is only present at a low concentration. Consequently, instead of copper cobalt or nickel will penetrate the cell. Since these metals are poor inhibitors of LDH and NADH oxidase growth inhibition is decreased.

Since hydrogen peroxyde is formed during the NADH oxidase reaction we wanted to investigate whether inhibition of this enzyme by copper is hydroxyl radical mediated. These radicals may be generated from hydrogen peroxyde and copper in a Fenton type reaction. Since iron(II) ions are known to generate hydroxyl radicals in the presence of hydrogen peroxyde we investigated the influence of these ions on NADH oxidase. If hydroxyl radicals would play a role in inhibition of NADH oxidase by copper, one would expect the IC_{50}-value for iron(II) ions to be smaller than or at least equal to the IC_{50}-value of copper(II) ions. However, NADH oxidase is much more sensitive to copper(II) ions than to iron(II) ions. Therefore it is not very likely that inhibition of NADH oxidase by copper is hydroxyl radical mediated.
Investigation of the influence of ligand **1** on both NADH oxidase and lactate dehydrogenase revealed that these enzymes are hardly inhibited by this compound. Furthermore it was shown that when copper and ligand are present in the assay simultaneously in a 1:2 ratio inhibition of both enzymes is markedly decreased as compared with inhibition by copper itself (figures I and II). The inhibitory activity of copper is decreased by copper binding compounds like 2,9-dimethyl-1,10-phenanthroline [13, 19] and ligand **1** (figures III and IV). These data prove that free copper is the most important species regarding enzyme inhibition. This is consistent with the proposed mechanism of action of antimycoplasmal activity of 2,2'-bipyridyl compounds in combination with small amounts of copper. In this mechanism of action free copper ions are the ultimate toxic species whereas 2,2'-bipyridyl ligands merely serve as carriers to facilitate transport of copper into the cell.

In the present investigation it was shown that both enzyme inhibition and antimycoplasmal activity are highly specific for copper. All other metals investigated i.e. cobalt, nickel, zinc and cadmium are not active against *Mycoplasma gallisepticum* and are less potent enzyme inhibitors. Due to their high affinity for ligand **1** and to their poor inhibitory action on NADH oxidase and LDH, cobalt and nickel are able to decrease antimycoplasmal activity of ligand **1** in the presence of relatively low copper concentrations.

Although inhibition of both NADH oxidase and lactate dehydrogenase by copper is decreased in the presence of ligand **1**, antimycoplasmal activity of copper is markedly increased by this ligand. These findings also are consistent with a mechanism of action in which copper is the ultimate toxic species and the ligand only serves as a carrier to transport copper into the cell.

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Chapter 8

DETERMINATION OF PROTON ASSOCIATION CONSTANTS AND COPPER(II) COMPLEX FORMATION CONSTANTS OF 1-AMINO-3-(2-PYRIDYL)ISOQUINOLINE AND 2-AMINO-1,10-PHENANTHROLINE DERIVATIVES

Introduction

As was shown in the previous chapters, antimycoplasmal activity of 1-amino-3-(2-pyridyl)-isoquinoline and 2-amino-1,10-phenanthroline derivatives is strongly dependent on the presence of copper. The same feature was found for several other 2,2'-bipyridyl derivatives [1-7].

It is known that compounds containing a 2,2'-bipyridyl moiety are able to chelate copper ions very well [8]. In fact it was shown that formation of a copper complex was an essential step in the whole process, eventually leading to mycoplasmal growth-inhibition.

The appearance of copper(I) complexes of 1,10-phenanthroline and 2,9-dimethyl-1,10-phenanthroline in growing cultures of *M. gallisepticum* has been demonstrated by Antic *et al.* [3]. Further evidence for the role of copper complexes in the growth-inhibiting process was provided by copper uptake studies by Smit and Gaisser [9-11]. Furthermore, structure-activity relationship studies revealed that antimycoplasmal activity of both 1-amino-3-(2-pyridyl)-isoquinoline and 2-amino-1,10-phenanthroline derivatives is dependent on their lipophilicity [12-14]. As transport across a lipophilic cell membrane is involved in the mode of action of these compounds one would expect such a dependency. Although electronic, and steric parameters as well, were included in structure-activity relationship studies of amides and amidines derived from both 1-amino-3-(2-pyridyl)isoquinoline and 2-amino-1,10-phenanthroline no significant correlation was found for these parameters in regard with antimycoplasmal activity. However, as a result of a quantitative structure-activity relationship study concerning the antimycoplasmal activity of compounds containing an ortho substituted 2,2'-bipyridyl moiety, an equation was obtained in which besides the hydrophobic fragmental value electronic and steric parameters were operating [15].

Since copper complexes are involved in the mechanism of action, we want to include physico-chemical parameters related to this complex formation in our structure-activity relationship study. Therefore, in the present investigation both proton association constants and copper complex formation constants of 1-amino-3-(2-pyridyl)isoquinoline and 2-amino-1,10-phenanthroline derivatives have been determined.

General considerations [16-18]

Potentiometry is one of the most popular methods for determination of stability constants because of its high accuracy and precision.

One of the main experimental approaches used for potentiometrical determination of stability constants is acid/base titration of a solution containing a constant total metal and total ligand concentration. By this method, the pH is measured as a function of the concentration of acid, or alkali added. Although pH measurements have been widely used to determine stability constants there are a number of limitations to the method.

In the first place it cannot be used under conditions of extreme pH. At high pH the concentration of free ligand is insensitive to pH change. At low pH the free ligand concentration becomes negligible owing to virtually complete protonation.

Furthermore, this method is inapplicable at very low total metal concentrations because under these conditions the degree of formation cannot be determined reliably.

The method is also inapplicable for very stable complexes, because in such cases the protons are unable to compete effectively with metal ions for the ligand. Since aqueous solutions always contain hydroxide ions and most metal ions form not only hydroxo-species but also polynuclear species, care must be taken either to work in sufficiently acidic solutions that hydroxo-complex formation is negligible or to take into account during analysis of the data.

Compounds containing a 2,2'-bipyridyl moiety can co-ordinate to a metal ion through more than one position; acting as multidentate ligands, they form chelate complexes. The thermodynamic equilibrium constant K^t for the equilibrium in which a metal ion (M) is the Lewis acid and a ligand is the Lewis base is known as the stability constant.

$$M + L \overset{K^t}{\rightleftharpoons} ML \quad *$$

* Throughout this chapter charges are omitted for the sake of simplicity, except when discussing specific metal ions or ligands.

When more than one ligand can co-ordinate to a certain metal ion, the formation of complexes occurs in a stepwise manner. Thus, K_1 and K_2 are known as stepwise stability constants.

$$M + L \overset{K_1{}^t}{\rightleftharpoons} ML \qquad\qquad ML + L \overset{K_2{}^t}{\rightleftharpoons} ML_2$$

$$K_1{}^t = \frac{a_{ML}}{a_M a_L} \qquad\qquad K_2{}^t = \frac{a_{ML_2}}{a_{ML} a_L}$$

a_X is the activity of species X, and is defined as the product of the molar concentration of X and its activity coefficient, γ_X.

The stability constant for the formation of ML_2 from M and L is called the overall stability constant β_2^t and $\beta_2^t = K_1^t \times K_2^t$.

As in practice many analytical techniques yield concentrations rather than activities, the thermodynamic stability constant is rewritten in terms of concentrations and activity coefficients:

$$K^t = \frac{a_{ML}}{a_M a_L} = \frac{[ML]}{[M]\,[L]} \times \frac{\gamma_{ML}}{\gamma_M \gamma_L}$$

The term $\gamma_{ML} / \gamma_M \gamma_L$ may be maintained effectively constant by, (a) having a large excess of an inert background electrolyte present (that is an electrolyte which does not react with any of the metal, ligand or metal-ligand species) and (b) using only low concentrations of metal and ligand so that any change in their concentrations as a result of their reaction together has an insignificant change on the overall ionic strength of the medium.

When the activity coefficients term is remained constant, the equation for the thermodynamic stability constant is reduced to the following equation:

$$K = \frac{[ML]}{[M]\,[L]}$$

K is known as the stoichiometric stability constant. Taking the above considerations into account, reliable data can be obtained by titration of a solution containing both a constant metal and a constant ligand concentration with an acid. As it is clear that protonation of the ligand is taking place, pK_a values of the ligands involved must be incorporated in the calculation of stability constants.

Therefore, these protonation constants have to be determined prior to the stability constants.

Proton-association constants

To avoid confusion about the symbols used to describe the equilibrium of protonation of the ligands under investigation, definitions are given first.

The equilibrium constant for protonation of a base is known as the proton association constant K_H^t.

$$L + H_3O^+ \rightleftharpoons LH^+ + H_2O$$

$$K_H^t = \frac{a_{LH^+}}{a_{H_3O^+} \times a_L}$$

The inverse of this equilibrium constant is known as the acid dissociation constant K_a.
Thus, $K_a = 1/K_H$ and $pK_a = \log K_H$.

As we are dealing with protonation of weak bases we prefer to use the term proton association constant. When the experimental conditions are properly chosen the activity coefficients will remain constant during the determination.

As was shown for stability constants, under these conditions the stoichiometric equilibrium constant K_H will be obtained instead of the thermodynamic equilibrium constant. This proton association constant K_H can be determined by potentiometric titration of the ligand with acid.

$$K_H = \frac{[LH^+]}{[H_3O^+]\,[L]}$$

As the base strength is markedly decreased after the first protonation step, further protonation of 2,2'-bipyridyl is not occuring under the applied conditions.

Since we want to investigate a possible relationship between these experimentally determined proton association constants of various ligands with their antimycoplasmal activity, it is neccessary to obtain these data under conditions comparable with those applied when establishing the biological activity.

As based on the salt concentration in this medium [12] the ionic strength of Adler medium was calculated to be 0.11, titrations were carried out in a 0.11 M potassium nitrate solution at 37°C.

Although water would be the preferred solvent, we had to use 50% (v/v) 1,4-dioxane/water instead, due to the low water solubility of the compounds under investigation. Consequently, the proton association constants obtained in this way are actually conditional proton association constants.

As the proton concentration in 50% (v/v) 1,4-dioxane/water does not give the correct pH when measured with a combined glass electrode calibrated in aqueous solution, the pH meter readings resulting from 50% (v/v) 1,4-dioxane/water containing 0.11 M potassium nitrate with different hydrogen ion concentrations (range 10^{-3}-10^{-6} M) were plotted against the calculated hydrogen ion concentration.

A good linear relationship was found:

$$pH_{obs} = 1.086\ (\pm 0.004)\ pH_{calc} - 0.118\ (\pm 0.017)$$
$$n = 20 \qquad r = 1.000 \qquad s = 0.006 \qquad F = 80898$$

Furthermore, the temperature dependency of the water dissociation constant K_w is given by the following equation [19]:

$$pK_{w(T)} = 14.9383\ (\pm 0.0025) - 4.167\ (\pm 0.019) \times 10^{-2}T + 1.622\ (\pm 0.03) \times 10^{-4}T^2$$
$$n = 13 \qquad r = 1.000 \qquad s = 0.004 \qquad F = 1.9 \times 10^5$$

In this equation T is the temperature in centigrades. So, from these two equations the dissociation constant of 50% (v/v) 1,4-dioxane/water at 37°C is calculated to be $pK_s = 14.672$.

Now, the conditional proton association constant K_H can be calculated from the following equations:

$$K_H = \frac{[LH^+]}{[L]\,[H_3O^+]} \qquad (1)$$

$$[L_T] = [L] + [LH^+] \qquad (2)$$

$$[H_3O^+] + [LH^+] = [OH^-] + [NO_3^-]_{added} \qquad (3)$$

$$[H_3O^+]\,[OH^-] = K_s \qquad (4)$$

In these equations $[LH^+]$ is the protonated ligand concentration, [L] is the free ligand concentration, $[L_T]$ is the total ligand concentration present in solution, $[H_3O^+]$ is the hydrated hydrogen ion concentration measured by a combined glass electrode, $[OH^-]$ is the free hydroxide concentration and $[NO_3^-]_{added}$ is the nitrate concentration as a consequence of the addition of nitric acid during titration.

From this set of equations an equation is derived in which K_H is a function of known parameters.

(3) and (4): $$[LH^+] = \frac{K_s}{[H_3O^+]} + [NO_3^-]_{added} - [H_3O^+] \qquad (5)$$

(2) and (5): $$[L] = [L]_T - \frac{K_s}{[H_3O^+]} - [NO_3^-]_{added} + [H_3O^+] \qquad (6)$$

(5), (6) and (1):

$$K_H = \frac{\dfrac{K_s}{[H_3O^+]} + [NO_3^-]_{added} - [H_3O^+]}{\left([L_T] - \dfrac{K_s}{[H_3O^+]} - [NO_3^-]_{added} + [H_3O^+]\right)\,[H_3O^+]}$$

For every point on the titration curve, K_H is calculated according to this equation from the weighed amount of ligand, the amount of titrant added and the experimentally determined pH.

After rejection of the statistical outliers the K_H value is obtained as the average value of this series of calculated values. K_H values presented in table I are the mean of three independent experiments.

Stability constants [16-18]

Before an attempt is made to quantify the equilibrium condition of any solution reaction, a clearly defined chemical model must be assumed.

For this purpose the nature of various chemical species in the solution have to be known.

The stoichiometry of species present in solution can be determined by the method of continuous variation (Job's Method) [18]. In this method a series of solutions is prepared in such a way that the sum of total metal and total ligand molar concentrations is constant.

Hence $[M_T] + [L_T] = C$. The absorbance of these solutions at a wavelength at which the molar extinction coefficients of the copper complex and the ligand are markedly different, is plotted against mole fraction of ligand, x. Subsequently the x_{max} value is determined from this plot as the mole fraction at which the absorbance is maximal.

Considering the formation of a complex ML_n, n is given by the following equation:

$$n = \frac{x_{max}}{1 - x_{max}}$$

As the basic structures of all compounds under investigation are very similar the method of continuous variation was applied to one of them viz. VUF 8514. It is assumed that the stoichiometry of the copper complexes formed from all other compounds is the same.

For the same reasons as described for determination of proton association constants, measurements of stability constants were performed in a 0.11 M potassium nitrate solution in 50% (v/v) 1,4-dioxane/water at 37°C. Consequently, also stability constants obtained are actually conditional stability constants. Data were obtained by titration of a solution containing a constant total metal and total ligand concentration with nitric acid.

In this system the following equilibria exist:

$$L + H_3O^+ \overset{K_H}{\rightleftharpoons} LH^+ + H_2O$$

$$M + L \overset{K_1}{\rightleftharpoons} ML$$

$$ML + L \overset{K_2}{\rightleftharpoons} ML_2$$

These equilibria are described by the following equations:

$$K_H = \frac{[LH^+]}{[L]\,[H_3O^+]} \qquad (7)$$

$$K_1 = \frac{[ML]}{[M]\,[L]} \quad (8) \qquad K_2 = \frac{[ML_2]}{[ML]\,[L]} \quad (9) \qquad \beta_2 = \frac{[ML_2]}{[M]\,[L]^2} \quad (10)$$

For calculation of stability constants the complex formation function n is introduced. This function n, defined as the average number of ligands, L, attached to the metal, M, may be written as

$$n = \frac{[\text{total ligand bound}]}{[\text{total metal}]} = \frac{[ML] + 2[ML_2]}{[M] + [ML] + [ML_2]} \qquad (11)$$

Substituting equations (8) and (10) in (11) yields

$$n = \frac{K_1[L] + 2\beta_2[L]^2}{1 + K_1[L] + \beta_2[L]^2} \qquad (12)$$

It is immediately apparent from equation (12) that now an equation is obtained in which n is solely dependent on the free ligand concentration, [L], and is independent of $[M_T]$, $[L_T]$ and the free metal ion concentration [M].
The free ligand concentration, [L], is obtained *via* the electroneutrality equation:

$$2\,[M^{2+}] + 2\,[ML^{2+}] + 2\,[ML_2^{2+}] + [H_3O^+] + [LH^+] = [OH^-] + [NO_3^-]_{added} + [X^-] \qquad (13)$$

In this equation X represents the anion of the metal salt (MX_2) used. As the concentration of this anion is equal to twice the sum of the concentrations of all metal containing species, equation (13) may be reduced to

$$[H_3O^+] + [LH^+] = [OH^-] + [NO_3^-]_{added} \qquad (13a)$$

or

$$[LH^+] = [OH^-] + [NO_3^-]_{added} - [H_3O^+] \qquad (13b)$$

Combination of equation (13b) with the equation for the water dissociation equilibrium (4) and equation (7), eventually gives an expression for [L]:

$$[L] = \frac{\frac{K_s}{[H_3O^+]} + [NO_3^-]_{added} - [H_3O^+]}{K_H\,[H_3O^+]} \qquad (14)$$

This value can be calculated from known constants, the amount of titrant added and the pH measured.

Graphical methods for the determination of stability constants K_1 and β_2 from the complex formation function n are very popular, as they make use of all data. Equation (12) can be arranged to give

$$\frac{n}{(1-n)\,[L]} = \frac{(n-2)}{(n-1)}\,[L]\beta_2 + K_1 \qquad (15)$$

If the left-hand side of equation (15) is plotted against $(n - 2 / n - 1)$ [L] a straight line results with slope β_2 and intercept K_1.

Whereas the free ligand concentration, [L], is calculated from equation (14), values for the complex formation function, n, are obtained from

$$n = \frac{\text{total ligand bound}}{\text{total metal}} = \frac{[L_T] - [L] - [LH^+]}{[M_T]} \qquad (16a)$$

or

$$n = \frac{[L_T] - [L] - K_H[L][H_3O^+]}{[M_T]} \qquad (16b)$$

Data were evaluated according to equation (15) with a linear regression program, especially made by us for this purpose. Without exception straight lines with excellent statistics ($r > 0.99$) were obtained for amidines derived from 1-amino-3-(2-pyridyl)isoquinoline and from 2-amino-1,10-phenanthroline. Values for K_1 and β_2 are presented in Table II, III and IV.

Experimental

Reagents

All 1-amino-3-(2-pyridyl)isoquinoline and 2-amino-1,10-phenanthroline derivatives were taken from the laboratory stock. Water was distilled from an all glass still after deionization over a mixed-bed ion exchange and 1,4-dioxane p.a. was purchased from J.T. Baker Chemicals, Holland. Standard buffers pH=7.00 (phosphate) and pH=4.00 (phthalate) were obtained from

J.T. Baker Chemicals Holland. Potassium nitrate p.a., nitric acid Titrisol and potassium hydroxide Titrisol were obtained from E. Merck, Darmstadt, BRD. Copper(II)acetate·H_2O p.a. was obtained from Ferak Berlin, BRD.

Disodium tetraborate p.a. for determination of the normality of standard nitric acid solutions, potassium hydrogenphthalate primary standard for standardisation of potassium hydroxide solutions and copper(II)nitrate·$3H_2O$ p.a. were purchased from J.T. Baker Chemicals, Holland. Copper nitrate solutions were standardized with EDTA [20], which was obtained from Sigma Chemical Company, USA.

Apparatus

The titration apparatus consisted of a water jacketed 100 ml vessel with a special cover in which appropriately located holes allowed the insertion of a Metrohm E202 combined glass electrode for registration of the pH, a burette inlet, and an inlet and outlet tube for nitrogen. Titrations were carried out while nitrogen gas was bubbling through the solution which was stirred magnetically.

Water from a Tamson TC3 waterbath, thermostatically maintained at 37.0 ± 0.01°C, was circulated through the jaketed beaker.

pH measurements were made with a Metrohm E580 digital ion activity meter and recorded as a function of the added volume nitric acid solution by means of a Mettler DV11 autoburette.

For determination of the amount of acid liberated as a consequence of copper complex formation a ligand was titrated with a standardized copper nitrate solution by means of a Mettler DV11 autoburette. These titrations were performed under the same conditions as described above.

The pH was held constant within 0.01 pH units by the addition of small quantities of potassium hydroxide solution, standardized with potassium hydrogen phthalate. The addition of potassium hydroxide was performed by a second Mettler DV11 autoburette, controlled by a Metrohm Impulsomat E473 autotitrator in combination with a Metrohm E202 combined glass electrode connected to a Metrohm E512 pH meter.

The volume of added amount of sodium hydroxide solution was recorded as a function of the added volume of the copper(II) solution on a Kipp BD30 x-y-t recorder.

As a control, the pH was measured with a Metrohm E202 combined glass electrode connected to a Metrohm E580 digital ion activity meter.

Procedure

Determination of proton association constants K_H

The glass electrode was calibrated daily with buffers of pH = 7.00 and pH = 4.00, respectively. For determination of proton association constants, a weighed quantity of the compound, corresponding to a 0.15 mM solution in a final volume of 50 mL was transferred to a dry titration vessel and 25.0 ml of 1,4-dioxane was added.
Subsequently 556 mg of potassium nitrate and 25.0 ml of water were added.
The mixture was stirred magnetically while nitrogen gas was passed through the solution.
When the temperature of the mixture had reached its final value of 37.0°C, the solution was titrated with 0.01 N HNO_3, standardized with disodium tetraborate. The total volume of titrant added was 1 mL. For each compound three titrations were performed and about 25 data points from each titration were used to calculate K_H.

Determination of stability constants K_1 and β_2

A weighed quantity of ligand, corresponding to a 0.35 mM solution in a final volume of 50 ml, was transferred to a dry titration vessel and 25.0 ml of 1,4-dioxane was added.
Subsequently, 556 mg of potassium nitrate, 10.0 ml of a standardized 1.1 mM copper nitrate solution and 15.0 ml of water were added. The mixture was stirred magnetically while nitrogen gas was passed through the solution.
When the temperature of the thermostatted mixture remained stable at 37°C, the solution was titrated with 0.1 N HNO_3, standardized with disodium tetraborate. In a typical experiment, the titration was stopped after the addition of 750 µl titrant.
For each compound three titrations were performed and about 30 data points from each titration were used for the calculation of stability constants.

Determination of the degree of deprotonation as a result of complex formation

A weighed quantity of ligand corresponding to a 0.07 mM solution in a final volume of 50 ml was transferred to a dry titration vessel and 25.0 ml of 1,4-dioxane was added.
Subsequently 556 mg of potassium nitrate and 25.0 ml of water were added.
The mixture was stirred magnetically while nitrogen gas was passed through and the pH of the solution was adjusted to the desired value. The temperature of the titration vessel was thermostatically maintained at 37.0 ± 0.01°C.
Subsequently, the mixture was titrated with a standardized 4.8 mM copper(II) solution, while keeping the pH constant with a standardized 9.6 mM potassium hydroxide solution.
Volumes of titrant added were less than 1 mL.

Synthesis of copper complexes

Copper(II) complex of N-[3-(2-pyridyl)isoquinolin-1-yl]-2-pyridinecarboxamidine (L).

A mixture of 650 mg of ligand (2 mmol) and 230 mg of $Cu(NO_3)_2 \cdot 3H_2O$ (0.94 mmol) in 200 mL of warm acetone was stirred for 1*h*. The precipitate was filtered off and subsequently crystallized from methanol, yielding 584 mg (71%) of dark-green needles of $CuL_2(NO_3)_2 \cdot 3H_2O$.

Elemental analysis (found/calculated): % C 54.75 / 54.95; % H 3.92 / 3.91; % N 19.17 / 19.22; % Cu 7.50 / 7.26; % H_2O 3.98 / 4.12.

Copper(II) complex of N-[3-(2-pyridyl)isoquinolin-1-yl]benzamide (L').

A mixture of 130 mg of ligand (0.4 mmol) and 40 mg of $Cu(OAc)_2 \cdot H_2O$ (0.2 mmol) in 100 mL of chloroform was stirred at room temperature for 4 *h*. The chloroform was removed by evaporation. The residual green solid was washed with methanol containing potassium hydroxide and subsequently crystallized from toluene, yielding 116 mg (82%) of fine, light-green needles of $Cu(L'_{-H})_2$.

Elemental analysis (found/calculated): % C 70.72 / 70.82; % H 3.98 / 3.96; % N 12.09/ 11.80; % Cu 8.61 / 8.92; % O 4.58 / 4.49.

Results and Discussion

Protonation constants

Protonation constants of both amides and amidines derived from either 1-amino-3-(2-pyridyl)isoquinoline or 2-amino-1,10-phenanthroline could be determined accurately by the potentiometric titration method described.

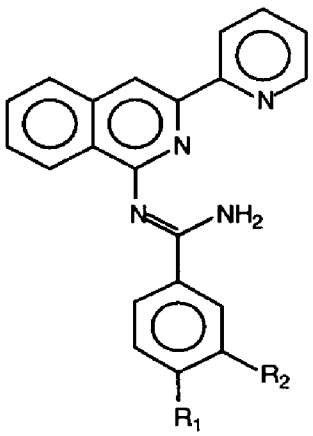
As can be seen from table I, protonation constants of N-[3-(2-pyridyl)isoquinolin-1-yl]-benzamidines are clearly influenced by the nature of the substituents in the benzamidine nucleus. Electron withdrawing substituents decrease the protonation constant of these bases, whereas electron donating substituents increase these constants.

Electron density and consequently affinity towards protons of nitrogen atoms involved in protonation is altered according to the electronic influence of substituents in the benzamidine nucleus.The effects observed can be fully explained by electronic features of these substituents, as indicated by the linear relationship which was found between the experimentally determined protonation constants and Hammett's $\sigma_{m,p}$:

$$\log K_H = 5.372\ (\pm 0.022) - 1.803\ (\pm 0.081)\ \sigma_{m,p}$$

n=8 r = 0.994 s = 0.058 F=492.389

Table I. Proton association constants (K_H) and copper complex stability constants (K_1, β_2) of N-[3-(2-pyridyl)isoquinolin-1-yl]benzamidines in 50% (v/v) 1,4-dioxane/water at 37°C with $\mu = 0.11$ (KNO_3).



Comp	R_1	R_2	log K_H (n = 3)	log K_1 (n = 3)	log β_2 (n = 3)	$\sigma_{m,p}$
VUF 8501	H	H	5.45±0.01	5.35±0.05	11.51±0.15	0
VUF 8514	H	CH_3	5.48±0.02	5.69±0.08	11.57±0.07	- 0.07
VUF 8503	CH_3	H	5.65±0.02	5.85±0.17	11.98±0.10	- 0.17
VUF 8510	H	OCH_3	5.22±0.02	5.40±0.05	11.77±0.03	0.12
VUF 8505	OCH_3	H	5.81±0.03	6.22±0.23	12.43±0.11	- 0.27
VUF 8513	H	Cl	4.64±0.02	- [a]	10.93±0.14	0.37
VUF 8470	Cl	H	5.00±0.02	5.54±0.16	11.21±0.13	0.23
VUF 8471	Cl	Cl	4.41±0.02	- [a]	10.01±0.06	0.52

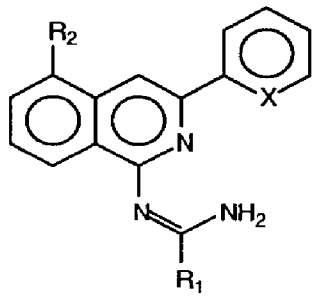
[a] Could not be determined significantly.

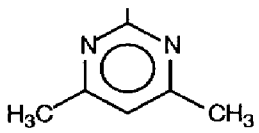
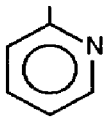
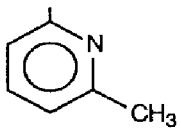
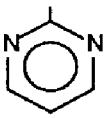
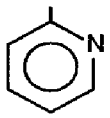
In table II protonation constants of some N-[3-(2-pyridyl)isoquinolin-1-yl]pyridine and N-[3-(2-pyridyl)isoquinolin-1-yl]pyrimidinecarboxamidines are presented.

Also for these compounds differences in protonation constants can be fully explained by considering electronic influences of the respective substituents. Due to the electron withdrawing effect of a 2-pyridine ring as compared to a phenyl ring, the protonation constant of VUF 8345 is considerable lower than of VUF 8501.

Comparison of protonation constants of VUF 8345 and VUF 8606 shows that substitution at position 5 of the isoquinoline nucleus and replacement of the 3-(2-pyridyl) ring by a 3-phenyl ring has little effect on the base strength of these compounds.

Table II. Proton association constants (K_H) and copper complex stability constants (K_1, β_2) of N-[3-(2-pyridyl)isoquinolin-1-yl]amidines and N-(5-methyl-3-phenylisoquinolin-1-yl)-2-pyridinecarboxamidine in 50% (v/v) 1,4-dioxane/water at 37°C with $\mu = 0.11$ (KNO_3).



Comp	R_1	R_2	X	log K_H (n = 3)	log K_1 (n = 3)	log β_2 (n = 3)
VUF 8613		H	N	5.67±0.02	6.24±0.02	12.20±0.06
VUF 8345		H	N	4.67±0.03	- [a]	10.16±0.08
VUF 8616		H	N	4.416±0.005	- [a]	9.93±0.09
VUF 8607		H	N	4.78±0.04	- [b]	- [b]
VUF 8606		CH_3	CH	4.61±0.04	- [c]	- [c]

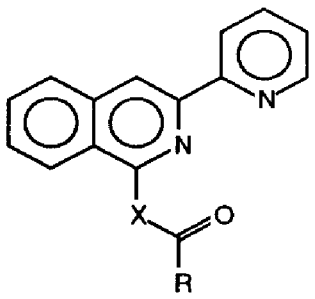
[a] Could not be determined significantly.

[b] Could not be determined due to deprotonation of ligand.

[c] Could not be determined due to precipitation.

Within these series only the protonation constant of VUF 8613 is substantially greater than of all other compounds. Due to the electron donating effect of the two methyl groups of the

Table III. Proton association constants (K_H) and copper complex stability constants (K_1, β_2) of N-[3-(2-pyridyl)isoquinolin-1-yl]amides and 1-phenacyl-3-(2-pryidyl)isoquinoline in 50% (v/v) 1,4-dioxane/water at 37°C with $\mu = 0.11$ (KNO_3).



Comp	R	X	log K_H (n = 3)	log K_1 (n = 3)	log β_2 (n = 3)
VUF 8346	CH_3	NH	4.22±0.02	4.18±0.03	9.35±0.02
VUF 8507	C_6H_5	NH	3.87±0.05	- [a]	- [a]
VUF 8516	C_6H_5	CH_2	3.44±0.06	- [a]	- [a]

[a] Could not be determined due to deprotonation of ligand.

pyrimidine nucleus, electron density on the nitrogen atoms and hence the affinity for protons is increased.

In general, amides are weaker bases than amidines as a consequence of the difference in electronegativity between nitrogen and oxygen [21,22].

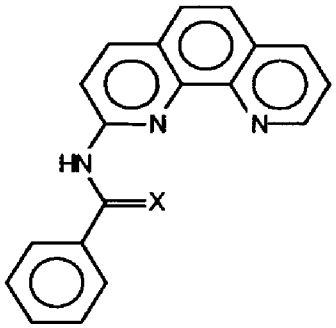
Comparison of the protonation constants of amides VUF 8507 and VUF 8827 (table III and IV respectively) with the corresponding amidines VUF 8501 and VUF 8837 (table I and IV respectively) shows that the same holds true for amides and amidines derived from 1-amino-3-(2-pyridyl)isoquinoline and 2-amino-1,10-phenanthroline.

Due to resonance benzamide VUF 8507 is a weaker base than the corresponding acetamide VUF 8346 as can be seen from table III. This difference in base strength is also observed for benzamide and acetamide itself [22].

Due to a higher degree of resonance stabilisation of the conjugate acid, 1,10-phenanthrolines are stronger bases than 2,2'-bipyridyls and 3-(2-pyridyl)isoquinolines. Comparison of protonation constants of 3-(2-pyridyl)isoquinolines VUF 8501 and VUF 8507 (table I and III respectively) with corresponding 1,10-phenanthrolines VUF 8837 and VUF 8827 (table IV) shows that also in these cases the latter are stronger bases.

As might be expected, base strength is decreased as a consequence of replacement of the

Table IV. Proton association constants (K_H) and copper complex stability constants (K_1, β_2) of 2-amino-1,10-phenanthroline derivatives in 50% (v/v) 1,4-dioxane/water at 37°C with μ = 0.11 (KNO_3).



Comp	X	log K_H (n = 3)	log K_1 (n = 3)	log β_2 (n = 3)
VUF 8827	O	4.152 ±0.002	4.06±0.01	9.11±0.03
VUF 8837	NH	6.80 ±0.05	7.49±0.01	13.44±0.11

nitrogen atom of the amide moiety of VUF 8507 by a carbon atom, resulting in ketone VUF 8516 (table III). In fact, base strength of the latter is almost reduced to the level of 3-(2-pyridyl)isoquinoline [23].

Summarizing it can be concluded from the results obtained that amides and amidines derived from 1-amino-3-(2-pyridyl)isoquinoline and 2-amino-1,10-phenanthroline are weak bases and consequently protonated only for a minor part at physiological pH. Because only electron density around the donor atoms affects the proton-ligand bond, differences in protonation constants can be explained very well by electronic influences. Furthermore it was shown that protonation constants of these compounds follow general trends.

Stability constants.

Before measurements for determination of stability constants are performed, the stoichiometry of the copper complex involved should be known first. This stoichiometry was determined by the method of continuous variation.

As can be seen from figure I the absorbance of $CuSO_4 \cdot 5H_2O$ / VUF 8514 mixtures reaches its maximal value at a mole fraction of ligand of 0.66. This means that a copper complex is formed in which the copper atom is surrounded by 2 ligand molecules.

Also elemental analysis of isolated copper complexes of VUF 8345 and VUF 8507 reveals that preferably a 1 : 2 (copper : ligand) complex is formed. As the colour of these copper complexes is green it is assumed that the oxidation state of the ligated copper is two. The observation that the colour of these copper(II) complexes is not changed in the presence of a reducing agent like hydroxylamine shows that the appearance of a copper(I) complex under aerobic conditions is not very likely.

Figure I. Absorbance at 401 nm of $CuSO_4 \cdot 5H_2O$ / VUF 8514 mixtures in 50% (v/v) 1,4-dioxane/water as function of mole fraction VUF 8514.

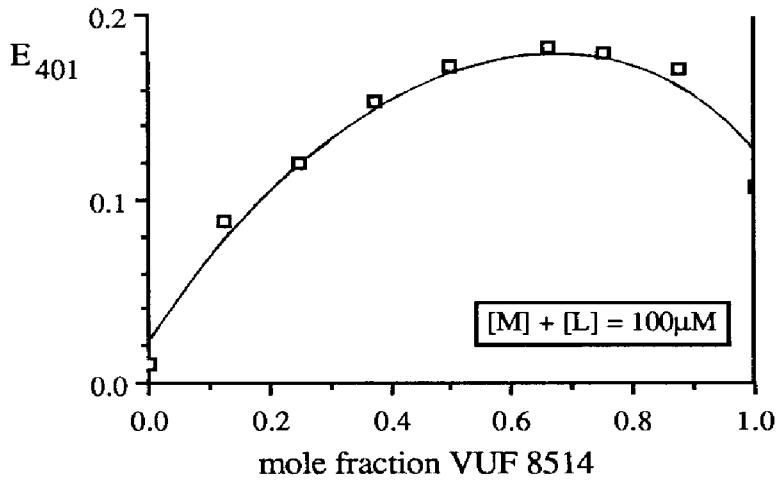
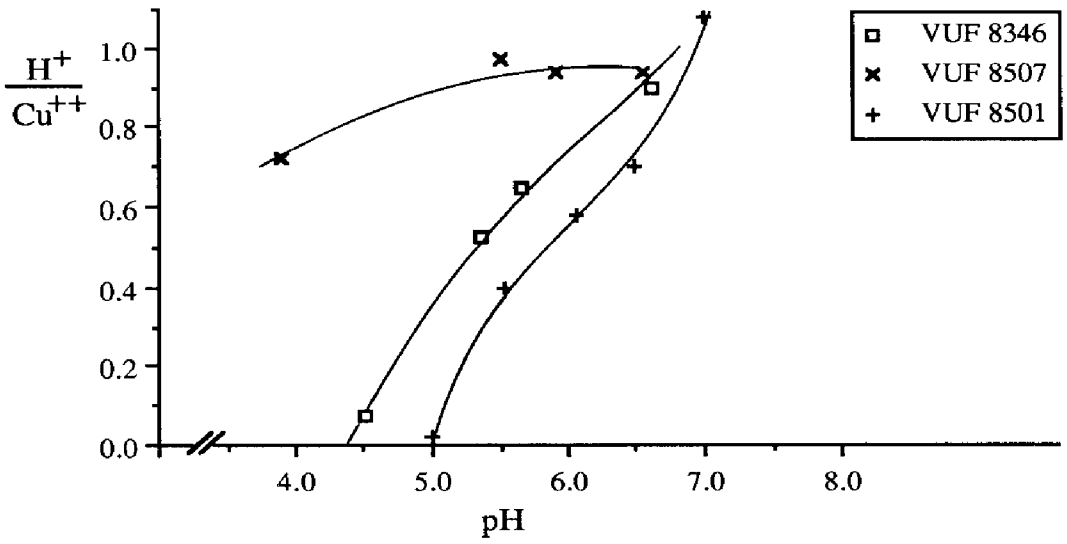


Figure II. pH dependency of the degree of deprotonation of some 1-amino-3-(2-pyridyl)isoquinoline derivatives.



Like protonation constants also copper complex stability constants of compounds under investigation could be determined accurately by the potentiometric titration method described. However, for the weaker complexes of VUF 8513, VUF 8471, VUF 8345 and VUF 8616 log K_1 could not be determined significantly. As a consequence of the applied experimental conditions the amount of the 1 : 1 complex present in solution, in these cases is too low to obtain reliable data for calculation of log K_1.

As we are mainly interested in the most stable complex viz. the 1 : 2 copper(II) complex, we didn't change experimental conditions in order to determine log K_1 values.

Due to precipitation during titration of VUF 8606 in the presence of copper stability constants of copper complexes of this compound could not be determined in this way.

Furthermore, it appeared also to be impossible to determine stability constants of the amide VUF 8507 and the ketone VUF 8516 by this procedure as a consequence of a phenomenon frequently encountered in studies dealing with formation of copper complexes [24-31]. These ligands are deprotonated as a consequence of the interaction with copper. Because of this, equations used to calculate stability constants from pH measurements are not valid anymore.

As we were able to determine stability constants of most of the compounds we present these data first, and will subsequently discuss problems encountered with specific compounds.

As was shown for protonation constants, stability constants of N-[3-(2-pyridyl)-isoquinolin-1-yl]benzamidines are dependent on the electronic influence of substituents in the benzamidine nucleus (table I). This observation is of course not surprising at all, since electron density on the nitrogen atoms is an important factor not only for protonation but for complex formation as well.

As can be seen from table I both stepwise stability constants are of the same order of magnitude, indicating that the same interactions are involved for both ligands [18].

Good linear relationships were found between experimentally determined stability constants of copper complexes of N-[3-(2-pyridyl)isoquinolin-1-yl]benzamidines and a parameter accounting for the electronic influence of a substituent like Hammet's $\sigma_{m,p}$.

$$\log K_1 = 5.637\ (\pm 0.088) - 1.426\ (\pm 0.518)\ \sigma_{m,p}$$

n=6 r=0.809 s=0.213 F=7.570

$$\log \beta_2 = 11.657\ (\pm 0.104) - 2.533\ (\pm 0.384)\ \sigma_{m,p}$$

n=8 r=0.937 s=0.276 F=43.435

In general stability constants of all compounds investigated follow the same trends as described for protonation constants. When the copper complex stability constants of the 3-(2-pyridyl)isoquinoline derivative VUF 8501 (Table I) are compared with the copper complex stability constants of the analogous 1,10-phenanthroline derivative VUF 8837 (Table IV), it is clear that the latter forms the more stable copper complexes.

At last, it can be seen from table IV that the amide VUF 8827 forms weaker complexes with copper than the corresponding amidine VUF 8837.

Furthermore, we have attempted to correlate log K_H values of all ligands investigated with their copper complex stability constants. As might be expected from the equations previously shown, a linear relationship appeared to exist between log K_H and the overall stability constant log β_2:

$$\log \beta_2 = 1.623\ (\pm 0.121) \log K_H + 2.817\ (\pm 0.625)$$

n=14 r=0.968 s=0.327 F=179.851

Correlations of the same type have been described for several other ligands [32,33].

The aim of these investigations was to obtain physico-chemical data related to protonation and copper complex formation of compounds under investigation in order to investigate if there is any correlation between these physico-chemical properties and antimycoplasmal activity of these compounds. It was shown for a series of substituted N-[3-(2-pyridyl)isoquinolin-1-yl]-benzamidines that a linear relationship appeared to exist between Hammett's $\sigma_{m,p}$ and both protonation and stability constants.

So, Hammett's $\sigma_{m,p}$ may be used in QSAR studies as a parameter accounting for differences in both protonation and stability constants. Although we had included electronic parameters like Hammett's $\sigma_{m,p}$ in our QSAR studies regarding antimycoplasmal activity, no significant correlation was found.

However, for a compound to possess antimycoplasmal activity stability constants are of decisive importance. As antimycoplasmal activity of these kind of compoounds is copper-dependent they should be able to bind copper, which is originally bound to growth-medium constituents [34]. In terms of stability constants this means that the product of the overall stability constant of an active compound and its concentration should be greater than the product of the overall stability constant of Adler medium (1.38×10^8) and the concentration of copper binding components in this medium (0.2M) [34].

Furthermore, it was established that copper binding to intracellular enzymes eventually caused growth-inhibition. So, ligated copper is transferred to these cytosolic components. This

means that the stability constant of the copper complex should be smaller than the stability constants of these enzymes.

Although stability constants of these specific enzymes are not known, very stable complexes have been described between copper(II) and various peptide chelates with stability constants varying from 10^{23}-10^{27} [35]. As stability constants of all compounds investigated are greater than 1.38×10^{8} and smaller than 10^{23}, it is clear that these compounds are able to transfer copper from growth-medium constituents to intracellular enzymes.

Because the cytosol is separated from the growth-medium by a cell membrane, transfer of ligated copper is further dependent on lipophilicity. In fact QSAR studies have revealed that antimycoplasmal activity of these compounds is dependent on lipophilicity of the ligands indeed [12,13].

Deprotonation

Finally we want to discuss the observation that some ligands appeared to be deprotonated as a consequence of formation of a copper complex. The amount of acid liberated as a result of the interaction of a ligand with copper could be determined by titration of this ligand with a copper nitrate solution, while keeping the pH of the solution at a constant value by adding a solution of potassium hydroxide simultaneously. The amount of hydroxide required to maintain the pH constant is directly proportional to the amount of acid liberated.

Table V. Degree of deprotonation at pH = 5.50 in 50% (v/v) 1,4-dioxane/water.

	H^+ / Cu^{2+}
VUF 8501[a]	0.40
VUF 8346[b]	0.60
VUF 8827[c]	0.57
VUF 8516[b]	0.94
VUF 8507[b]	0.98

[a]See for the structural formula of this compound table I.

[b]See for the structural formula of this compound table III.

[c]See for the structural formula of this compound table IV.

As can be seen from table V all kinds of ligands appeared to be deprotonated when treated with copper nitrate although the degree of deprotonation at pH=5.50 is different for each ligand. For

obvious reasons amide VUF 8507 is more acidic than the corresponding amidine VUF 8501. However, it appeared to be impossible to abstract the amide proton with concentrated hydroxide.

As a consequence of resonance stabilisation the aromatic amide VUF 8507 is more acidic than the aliphatic amide VUF 8346. Resonance stabilisation also accounts for the acidity of ketone VUF 8516.

As can be seen from figure II the degree of deprotonation is pH dependent. During determination of stability constants pH varied from about 4.5 to 2.7. Within this pH range deprotonation is not appearing for VUF 8346 and VUF 8501. This means that stability constants presented in tables I-IV are reliable data, although it is very likely that at physiological pH deprotonated ligands bind copper.

Copper complexes of VUF 8345 and of VUF 8507 were synthesized according to a procedure described by Yamada *et al.* [30]. As has been described by these authors for complexes of 6,6'-bis(acylamino)-2,2'-bipyridine complexation of VUF 8346 with copper(II)nitrate gave a non-deprotonated complex, whereas complexation of VUF 8507 with copper(II)acetate gave a deprotonated complex. It is noteworthy that the latter could be crystallized from toluene, indicating that this complex is rather lipophilic.

So, it is likely that the compounds under investigation form copper(II) complexes in which the central copper atom is octahedrally surrounded by two deprotonated ligands. In such a complex each ligand might co-ordinate copper *via* the two heterocyclic nitrogen atoms and the oxygen atom of the deprotonated amide moiety or the nitrogen atom of the deprotonated amidine moiety, respectively. Formation of such neutral, lipophilic complexes may also account for the remarkably high antimycoplasmal activity of amides and amidines derived from 1-amino-3-(2-pyridyl)isoquinoline and 2-amino-1,10- phenanthroline.

Conclusions

In this study proton association constants and copper(II) complex formation constants of 1-amino-3-(2-pyridyl)isoquinoline and 2-amino-1,10-phenanthroline derivatives have been determined accurately by a potentiometric titration method. All compounds appeared to be weak bases, forming stable copper(II) complexes in which the central copper ion is surrounded by two ligand molecules. Dependent on pH ligands could be deprotonated as a consequence of copper complex formation, resulting in neutral complexes.

Both protonation and copper complex stability constants appeared to be strongly dependent on electronic influences. In fact, for a series of substituted N-[3-(2-pyridyl)isoquinolin-1-yl]-benzamidines a good correlation was found between these experimentally determined constants and Hammett's $\sigma_{m,p}$. Furthermore, a linear relationship was established between protonation

constants and copper complex stability constants of all compounds investigated.

As electronic influences account for differences in both protonation and copper complex stability constants, electronic parameters like Hammett's $\sigma_{m,p}$ may be used to investigate the influence of these physico-chemical properties on antimycoplasmal activity.

Previous QSAR studies have already shown that no significant correlation between Hammet's $\sigma_{m,p}$ and antimycoplasmal activity appeared to exist. However, stability constants of active compounds should be in between stability constants of intracellular components in order to transfer copper from the growth-medium to intracellular peptides. It is clear from this study that these requirements are met by all compounds investigated.

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SUMMARY

The investigations described in this thesis were aimed at the development of compounds, which are highly active against *Mycoplasma gallisepticum*.
In general, Mycoplasmas are known to be causative agents of many infectious diseases not only in plants and animals but in men as well. *Mycoplasma gallisepticum* is the causative agent of chronic respiratory disease in poultry.

Mycoplasmas are procaryotes, which are characterized by the lack of a rigid cell wall. Accordingly, the individual organisms are pleomorphic and vary in shape from coccoid to filamentous to helical. With their diametrical size of 100-1000 nm they are the smallest free-living organisms capable of self-reproduction.

As these microorganisms possess no cell wall they are insensitive for the action of penicillins. Broad-spectrum antibiotics from the small polyene type (34-37 carbon atoms) and the tetracycline type are inhibitory to mycoplasmas *in vitro* as well as *in vivo*. Unfortunately, these broad-spectrum antibiotics induce resistance rapidly. Tylosin, a mocrolide antibiotic, is often used in therapy of mycoplasmal infections in poultry.

In 1975 Van der Goot described the synthesis and antimycoplasmal activity of 1-amino-3-(2-pyridyl)isoquinoline. The presence of a 2,2'-bipyridyl skeleton appeared to be essential with regard to this activity.

Subsequent investigations by Pijper revealed that antimycoplasmal activity of 1-amino-3-(2-pyridyl)isoquinoline and related compounds was dependent on the presence of copper. A QSAR study revealed that compounds containing a cis-coplanar, ortho-substituted 2,2'-bipyridyl moiety and a certain degree of lipophilicity were active against *M.gallisepticum* in the presence of a small, as such non-toxic, amount of copper. As structural requirements for antimycoplasmal activity were identical with those required for formation of stable copper(I) complexes, it was concluded that the lipophilic copper(I) complexes of the 2,2'-bipyridyl analogues investigated were the active species with respect to antimycoplasmal activity.

Investigations into the mode of action of these copper complexes revealed that copper itself is the ultimate toxic agent, whereas ligands facilitate copper transport across the cell membrane through formation of lipophilic complexes. The toxicity of copper is most probably based on the inhibition of enzymes involved in the energy providing metabolism like NADH oxidase and lactate dehydrogenase.

As amides and amidines derived from 4-amino-2-(2-pyridyl)quinazoline appeared to be more active against *M.gallisepticum* in the presence of copper than tylosin and the parent compound, the copper-dependent antimycoplasmal activity of amides and amidines of the related 1-amino-3-(2-pyridyl)isoquinoline has been investigated.

After the presentation of a survey of various biological activities of copper complexes in chapter 2, the synthesis and copper-dependent antimycoplasmal activity of both aliphatic and aromatic amides derived from 1-amino-3-(2-pyridyl)isoquinoline has been described in chapter 3. These amides appeared to be potent antimycoplasmal agents however, in the presence of copper only, with minimal inhibitory concentrations (mic) ranging from 0.1-2 μM. Acylation of 1-amino-3-(2-pyridyl)isoquinoline resulted in more active compounds, with the most active compound being as active as tylosin.

Furthermore a quantitative structure-activity relationship study revealed a good correlation between antimycoplasmal activity and the hydrophobic fragmental value of the amide residue. This dependency on hydrophobic fragmental values is parabolic in nature, indicating that an optimal lipophilicity for antimycoplasmal activity exists.

In chapter 4 investigations on the influence of structural modifications of these amides on antimycoplasmal activity have been described. The amide moiety appeared to be essential for the high activity of these compounds. When the nitrogen atom of the amide moiety was replaced by a carbon atom, antimycoplasmal activity was markedly decreased. Although ortho-substitution of the 2,2'-bipyridyl moiety appeared to increase antimycoplasmal activity of compounds investigated by Pijper, antimycoplasmal activity of amides of 1-amino-3-(2-pyridyl)isoquinoline was not increased as a consequence of the introduction of a substituent at the ortho position of the 3-(2-pyridyl) ring, indicating that in this case probably another kind of copper complex was formed.

In addition to the amides, the analogous amidines derived from 1-amino-3-(2-pyridyl)-isoquinoline were synthesized and tested for their antimycoplasmal potency. In the presence of copper these compounds appeared to be even more active than the corresponding amides, with mic values in the nanomolar range. Even without the addition of extra copper some of these compounds show complete growth-inhibition in the micromolar range.
Antimycoplasmal activity of amidines derived from 1-amino-3-(2-pyridyl)isoquinoline appeared to be dependent on lipophilicity as was found for the structurally related amides and this dependency was parabolic in nature, which means that for these amidines too an optimal lipophilicity for antimycoplasmal activity exists.

Although a great increase of antimycoplasmal activity is already obtained by structure optimization within a series of 1-amino-3-(2-pyridyl)isoquinoline derivatives, the attention was subsequently focussed on the corresponding 2-amino-1,10-phenanthroline derivatives in an attempt to obtain even more active compounds. For these compounds too, the presence of a small amount of copper appeared to be a necessary prerequisite for antimycoplasmal activity.

All derivatives of 2-amino-1,10-phenanthroline are more active against *M.gallisepticum* than the parent compound and the amides are more active than the corresponding amidines. The

most active compounds within this series are as active as the most active compound thusfar investigated. For the amides a good linear correlation was found between antimycoplasmal activity and lipophilicity of these ligands. From this relationship it is clear that for this series of compounds antimycoplasmal activity is increased upon a decrease of the hydrophobic fragmental value of the substituents considered. Due to the limited number of amidines available no quantitative structure-activity realtionship could be established for these compounds.

Subsequently the influence of various transition metal ions on both growth and enzyme activity of *M.gallisepticum* has been investigated. It was shown that cobalt, nickel, zinc and cadmium are not active against *M.gallisepticum* and are less potent enzyme inhibitors as compared to copper. Due to their high affinity for a 2,2'-bipyridyl type ligand and to their poor inhibitory action on NADH oxidase and lactate dehydrogenase, cobalt and nickel are able to decrease antimycoplasmal activity of such a ligand in the presence of relatively low copper concentrations. Although inhibition of the aforementioned enzymes by copper is decreased in the presence of a 2,2'-bipyridyl type ligand, antimycoplasmal activity of copper is markedly increased by this ligand.
These findings are consistent with a mechanism of action in which copper is the ultimate toxic species and the ligand only serves as a carrier to transport copper into the cell.

Since copper complexes are involved in the mechanism of action, the formation constants of the copper complexes of 1-amino-3-(2-pyridyl)isoquinoline and 2-amino-1,10-phenanthroline derivatives have been determined in order to investigate if there is a relationship between antimycoplasmal activity and these copper complex formation constants. Additionally, the proton association constants of the same derivatives have been determined.

The compounds investigated appeared to form stable copper(II) complexes, in which the central copper(II) ion is surrounded by two ligand molecules. Both protonation constants of these ligands and the copper(II) complex formation constants could be explained very well by electronic influences. Within a series of closely related compounds a good linear correlation was found between these constants and an electronic parameter like Hammett's σ. As it was established that antimycoplasmal activity was not dependent on such electronic parameters it is concluded that within these series of compounds lipophilicity is the determinant factor for antimycoplasmal activity.

It was found that some ligands were deprotonated upon copper complex formation, resulting in the formation of neutral lipophilic complexes. This deprotonation appeared to be pH-dependent. It is assumed that all compounds are deprotonated at physiological pH as a consequence of copper(II) complex formation. Formation of neutral, lipophilic copper(II) complexes may account for the tremendous increase in antimycoplasmal activity of these compounds as compared to the respective parent compounds i.e. 1-amino-3-(2-pyridyl)-

isoquinoline and 2-amino-1,10-phenanthroline.

SAMENVATTING

Het in dit proefschrift beschreven onderzoek was gericht op de ontwikkeling van verbindingen met een hoge activiteit tegen *Mycoplasma gallisepticum*. Mycoplasma's zijn algemeen bekend als de veroorzakers van vele infectie ziekten, zowel bij planten als bij dieren en mensen. *M.gallisepticum* veroorzaakt een aandoening van de luchtwegen bij pluimvee. Mycoplasma's zijn prokaryoten, die gekenmerkt worden door het ontbreken van een celwand. Als gevolg daarvan zijn de individuele organismen pleomorf. Met een diameter van 100-1000 nm zijn ze de kleinste vrij levende organismen die in staat zijn zichzelf voort te planten.

Omdat ze geen celwand bezitten zijn ze ongevoelig voor de werking van penicillines. Verschillende breed-spectrum antibiotica zoals polyenen en tetracyclines remmen, zowel *in vitro* als *in vivo* , de groei van mycoplasma's. Helaas induceren deze breed-spectrum antibiotica snel resistentie. Tylosine is een macrolide antibioticum dat vaak toegepast wordt in de behandeling van mycoplasma infecties in pluimvee.

In 1975 werd de synthese en antimycoplasma activiteit van 1-amino-3-(2-pyridyl)-isochinoline beschreven door Van der Goot. De aanwezigheid van een 2,2'-bipyridyl skelet bleek essentiëel te zijn met betrekking tot deze activiteit.

Een vervolg onderzoek door Pijper toonde aan dat de antimycoplasma activiteit van 1-amino-3-(2-pyridyl)isochinoline en aanverwante verbindingen afhankelijk is van de aanwezigheid van koper. Een QSAR studie toonde aan dat verbindingen met een cis-coplanair ortho gesubstitueerd 2,2'-bipyridyl deel en een zekere mate van lipofiliteit actief zijn tegen *M.gallisepticum* in aanwezigheid van een kleine, op zichzelf niet toxische, hoeveelheid koper.
Gezien de overeenkomst tussen de structuureisen voor antimycoplasma activiteit en voor de vorming van stabiele koper(I)complexen, werd geconcludeerd dat de lipofiele koper(I)-complexen van de onderzochte 2,2-bipyridyl analoga de actieve verbindingen waren met betrekking tot antimycoplasma activiteit.

Onderzoek naar het werkingsmechanisme van deze kopercomplexen toonde aan dat koper zelf het uiteindelijk toxisch bestanddeel vormde, terwijl de liganden het transport van koper over de celmembraan vergemakkelijkten door de vorming van lipofiele complexen.
De toxiciteit van koper wordt waarschijnlijk veroorzaakt door de remming van enzymen, die betrokken zijn bij het energie leverende metabolisme zoals NADH oxidase en lactaat-dehydrogenase. Omdat amides en amidines afgeleid van 4-amino-2-(2-pyridyl)chinazoline in de aanwezigheid van koper actiever bleken te zijn tegen *M.gallisepticum* dan tylosine en de moeder verbinding, werd de koperafhankelijke antimycoplasma activiteit van amides an amidines van het verwante 1-amino-3-(2-pyridyl)isochinoline onderzocht.

Na het geven van een overzicht van verschillende biologische activiteiten van

kopercomplexen in hoofdstuk 2, wordt de synthese en koperafhankelijke antimycoplasma activiteit van zowel alifatische als aromatische amides afgeleid van 1-amino-3-(2-pyridyl)-isochinoline beschreven in hoofdstuk 3.
Deze amides bleken alleen in de aanwezigheid van koper zeer potente antimycoplasma verbindingen te zijn met minimaal groeiremmende concentraties (mgc) variërend van 0.1-2 μM. Acylering van 1-amino-3-(2-pyridyl)isochinoline leverde meer actieve verbindingen op, terwijl de meest actieve verbinding net zo actief is als tylosine. Verder toonde een onderzoek naar kwantitatieve structuur-activiteit relaties aan dat er een goede correlatie bestaat tussen antimycoplasma activiteit en de hydrofobe fragment waarde van het amide gedeelte. Deze afhankelijkheid was parabolisch van aard, wat er op duidt dat er een voor antimycoplasma activiteit optimale lipofiliteit bestaat.

In hoofdstuk 4 wordt het onderzoek naar de invloed van structuur variaties van deze verbindingen op de antimycoplasma activiteit beschreven. Het amide deel blijkt essentiëel te zijn voor de hoge activiteit van deze verbindingen. Wanneer het amide stikstof atoom wordt vervangen door een koolstof atoom, neemt de antimycoplasma activiteit aanzienlijk af. Hoewel ortho substitutie van het 2,2'-bipyridyl gedeelte in de door Pijper onderzochte verbindingen de antimycoplasma activiteit bleek te verhogen, werd de antimycoplasma activiteit van amides afgeleid van 1-amino-3-(2-pyridyl)isochinoline daarentegen niet verhoogd door de introductie van een ortho substituent in de 3-(2-pyridyl) ring, wat aangeeft dat er in dit geval waarschijnlijk een ander kopercomplex wordt gevormd.

Naast de amides zijn de analoge amidines afgeleid van 1-amino-3-(2-pyridyl)isochinoline gesynthetiseerd en getest op hun antimycoplasma activiteit. Deze verbindingen bleken in de aanwezigheid van koper zelfs actiever te zijn dan de overeenkomstige amides, met mgc waarden in het nanomolair bereik. Zelfs zonder toevoeging van extra koper bleken sommige verbindingen in micromolair concentraties de groei volledig te remmen. Net zoals gevonden was voor de structuur verwante amides, bleek de antimycoplasma activiteit van amidines afgeleid van 1-amino-3-(2-pyridyl)isochinoline afhankelijk te zijn van de lipofiliteit en deze afhankelijkheid was parabolisch van aard, hetgeen betekent dat ook voor deze amidines een, voor antimycoplasma activiteit, optimale lipofiliteit bestaat.

Hoewel er reeds een aanzienlijke toename in antimycoplasma activiteit is verkregen door structuuroptimalisatie van een serie 1-amino-3-(2-pyridyl)isochinoline derivaten, werd in een poging om nog actievere verbindingen te verkrijgen de aandacht gericht op de overeenkomstige 2-amino-1,10-fenanthroline derivaten. Ook voor deze verbindingen bleek de aanwezigheid van een kleine hoeveelheid koper een voor antimycoplasma activiteit noodzakelijke voorwaarde te zijn. Alle derivaten van 2-amino-1,10-fenanthroline zijn actiever tegen *M.gallisepticum* dan de moeder verbinding, terwijl de amides actiever zijn dan de overeenkomstige amidines. De meest

actieve verbindingen binnen deze serie zijn net zo actief als de tot dan toe meest actieve verbinding. Voor de amides werd een goede lineaire correlatie gevonden tussen antimycoplasma activiteit en lipofiliteit. Uit de gevonden correlatie blijkt de antimycoplasma activiteit toe te nemen wanneer de hydrofobe fragment waarde van de substituenten afneemt. Gezien het geringe aantal beschikbare amidines kon er voor deze verbindingen geen kwantitatieve structuur-activiteit relatie worden vastgesteld.

Vervolgens werd de invloed van verschillende overgangsmetaalionen op zowel de groei als de enzymactiviteit van *M.gallisepticum* onderzocht. Zowel kobalt als nikkel, zink en cadmium bleken niet actief tegen *M.gallisepticum* en zijn minder potente enzymremmers dan koper. Vanwege hun hoge affiniteit voor liganden van het 2,2'-bipyridyl type en hun geringe remmende werking op NADH oxidase en lactaatdehydrogenase zijn kobalt en nikkel in staat om de antimycoplasma activiteit van een dergelijk ligand te verlagen in aanwezigheid van een relatief lage koperconcentratie.

Hoewel de remming van bovengenoemde enzymen door koper verlaagd wordt door de aanwezigheid van een ligand van het 2,2'-bipyridyl type, wordt de antimycoplasma activiteit van koper juist aanzienlijk verhoogd door een dergelijk ligand. Dit is consistent met een werkingsmechanisme waarin koper het uiteindelijk toxisch bestanddeel is en waarin het ligand dient als transportmiddel om koper in de cel te brengen.

Aangezien kopercomplexen betrokken zijn bij het werkingsmechanisme, zijn de vormingsconstanten van de kopercomplexen van 1-amino-3-(2-pyridyl)isochinoline en 2-amino-1,10-fenanthroline derivaten bepaald, om te onderzoeken of er een relatie bestaat tussen antimycoplasma activiteit en deze kopercomplex vormingsconstanten. Bovendien zijn de proton associatieconstanten van dezelfde derivaten bepaald.

De onderzochte verbindingen blijken stabiele koper(II)complexen te vormen, waarbij het centrale koper(II)ion wordt omgeven door twee ligand moleculen. Zowel proton associatieconstanten van deze liganden als de koper(II)complex stabiliteitsconstanten kunnen volledig verklaard worden op basis van electronische invloeden. Binnen een serie van nauw verwante verbindingen werd een goede correlatie gevonden tussen deze constanten en een electronische parameter als Hammett's σ.

Bovendien werd gevonden dat sommige liganden gedeprotoneerd werden als gevolg van de vorming van een kopercomplex, hetgeen resulteert in de vorming van neutrale, lipofiele complexen. Deze deprotonering bleek pH afhankelijk te zijn. Aangenomen wordt dat alle onderzochte verbindingen bij fysiologische pH gedeprotoneerd worden als gevolg van koper(II)complex vorming.

Vorming van neutrale, lipofiele koper(II)complexen kan de enorme toename in antimycoplasma activiteit van deze verbindingen in vergelijking met de uitgangsverbindingen

1-amino-3-(2-pyridyl)isochinoline en 2-amino-1,10-fenanthroline verklaren.

CURRICULUM VITAE

Marcel de Zwart was born in 1958 on March 11, at The Hague, The Netherlands.
He graduated from secondary school (Gymnasium-B) in 1976. In the same year he entered the Faculty of Science and Mathematics of the Catholic University at Nijmegen, where he acquired his B.Sc. in Chemistry in 1979.
After studying Chemistry with Medicinal Chemistry as principal subject and Organic chemistry and Microbiology as subsidiary subjects, he received his M.Sc. in Chemistry in 1984.
Additionally, he received his qualification to teach Chemistry and he attended a course in Environmentology.
In September 1984 he joined the Department of Pharmacochemistry of the Free University at Amsterdam as a scientific research assistant. From that time until June 1989 the investigations described in this thesis were carried out in this department.

LIST OF PUBLICATIONS

Marcel AH de Zwart, Henk van der Goot and Henk Timmerman,
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MAH de Zwart, H van der Goot, H Timmerman,
Structure Optimization of Amides and Amidines Derived from 1-Amino-3-(2-pyridyl)-isoquinoline Concerning Antimycoplasmal Activity. In: *Biology of Copper Complexes* (Ed. John RJ Sorenson), p.509. The Humana Press, Clifton, New Jersey, USA, 1987.

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Characterization of Peripheral Benzodiazepine Binding Sites in Bovine Lung.
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SLOTWOORD

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STELLINGEN

1. Het verdient aanbeveling om bij de synthese van 1-amino-3-(2-pyridyl)-isochinoline uit 2-methylbenzonitril en 2-pyridinecarbonitril equimolaire hoeveelheden van beide reaktanten te gebruiken.

   Van der Goot H en Nauta WTh, *Chim Ther* 7: 185, 1972.
   Dit proefschrift

2. Het door Gaisser voorgestelde mechanisme van de antimycoplasma aktiviteit van 1-amino-3-(2-pyridyl)isochinoline en 2-amino-1,10-fenantroline geldt niet voor van deze verbindingen afgeleide amides en amidines.

   Gaisser H-D, Proefschrift 'The role of copper in the mode of action of 2,2'-bipyridyl analogues with antimycoplasmal activity-a (Q)SAR study', Vrije Universiteit, Amsterdam, 1985.
   Dit proefschrift

3. Bij de bestudering van struktuur-aktiviteit relaties met betrekking tot de biologische aktiviteit van kopercomplexen kan de invloed van metaalcomplex-vorming op de biologische aktiviteit adekwaat beschreven worden door elektronische parameters.

   Dit proefschrift

4. Bij het uitsluiten van metaalcomplexvorming als een mogelijke verklaring voor de fungicide aktiviteit van 2,9-dimethyl-1,10-fenantroline gaan Shulman en Dwyer voorbij aan de mogelijke vorming van het 2,9-dimethyl-1,10-fenantroline koper(I)complex.

   Shulman A en Dwyer FP in 'Chelating Agents and Metal Chelates' van Dwyer FP en Mellor DP. Academic Press, New York, 1964.

5. Het is onwaarschijnlijk dat de door Løvstad voorgestelde binding van 2,9-dimethyl-1,10-fenantroline, 1,10-fenantroline en 2,2'-bipyridyl aan ceruloplasmine plaats vindt via interaktie met het gereduceerde type-4 koperion.

   Løvstad RA, *Int J Biochem* **20**: 117, 1988.

6. De voorspellende waarde van *in vitro* methodes voor de bepaling van superoxide dismutase aktiviteit van kopercomplexen *in vivo* dient niet overschat te worden.

Czapski G en Goldstein S, *Free Rad Res Comm* **4**: 225, 1988.

7. Het door Mohindru *et al.* gevonden verschil in antitumor aktiviteit van 2,9-dimethyl-1,10-fenantroline en 1,10-fenantroline in het door hen gebruikte intakte celsysteem moet verklaard worden uit de vorming van verschillende kopercomplexen.

Mohindru A, Fisher JM en Rabinovitz M, *Biochem Pharm* **32**: 3627, 1983.

8. Het wetenschappelijk onderwijs zou gebaat zijn met scholing van zijn docenten in didaktiek .

9. Loodvrije benzine is niet milieuvriendelijk.

10. De schier oneindige mogelijkheden van een tekstverwerker maken het werken ermee eindeloos.

Amsterdam, 17 november 1989 Marcel de Zwart

Stellingen

behorend bij het proefschrift

'Synthesis and Copper-Dependent Antimycoplasmal Activity

of 3-(2-Pyridyl)isoquinoline and 1,10-Phenanthroline Derivatives'

van Marcel A.H. de Zwart.